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THE QUANTITATIVE SIGNIFICANCE OF THE LYMPHATIC PATHWAY IN TRANSPORT OF ABSORBED FATTY ACIDS*

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The claim that the major portion of absorbed fat passes into the lymph stream is based on the classical experiment of Munk and Rosenstein carried out more than 50 years ago on a patient suffering from elephantiasis, in whom a lymphatic fistula developed in the left leg (1). From such a fistula, these workers were able to recover as much as 60 per cent of the fat of an ingested meal. Such a high recovery was not observed by later investigators in animal experiments. Eckstein, working with dogs fed emulsified olive oil, reported recoveries in thoracic duct lymph as high as 21 per cent in 12 hours, and 17 per cent in 6 hours (2). When he fed the dogs oleic and palmitic acids, the recoveries were much lower. Since the dogs were anesthetized throughout the entire period of lymph collection, Eckstein was careful to point out that he did not expect fat absorption to be as good as it would have been under more normal conditions. Such low recoveries of absorbed fat in thoracic duct lymph of the dog were also observed by Little and Robinson (3).

The availability of C^{14} -labeled fatty acids, together with the development by Bollman *et al.* (4) of a method for collecting lymph from the unanesthetized rat, made possible a reinvestigation of this problem.

EXPERIMENTAL

Collection of Lymph

Male rats of the Long-Evans strain, not fasted before the operation, were used in this study. The procedures used for obtaining samples from the thoracic duct and intestinal lacteals were modifications of those described by Bollman, Cain, and Grindlay (4).

Thoracic Duct—The peritoneal cavity was opened by a mid-line incision extending from the xiphisternal junction to a point about 3 cm. from the symphysis pubis. With the aid of the dissecting microscope, the thoracic duct was carefully freed from the dorsal aorta. A polythene cannula was

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inserted through the left lateral body wall just caudal to the costal margin, and introduced into the duct, where it was securely fixed by two ligatures. The muscular wall and skin were then closed by continuous suture. The entire operative procedure was usually completed in 25 minutes, during which the rats were under ether anesthesia.

Intestinal Lymphatic—The two intestinal lymphatic trunks pass along the superior mesenteric artery and are connected by anastomotic vessels. To obtain intestinal lymph, the polythene cannula was inserted into the larger trunk, and the smaller trunk was ligated.

Lymph collection from the unanesthetized animal was made possible by keeping the rat in a restraining cage similar in design to that described by Bollman (5). The rats with thoracic duct fistulae had free access to food and 1 per cent NaCl solution for the duration of the experiment. Those from which intestinal lymph was collected had free access to food throughout. Before administration of the labeled fatty acid, a 1 per cent NaCl solution was available for drinking purposes; thereafter, tap water was made available.

Administration of Labeled Fatty Acids

Palmitic acid labeled with C^{14} at the carboxyl position was synthesized by the method described elsewhere (6). It was administered either as the tripalmitin or as the free fatty acid dissolved in 0.5 cc. of corn oil. The mixtures were brought to body temperature and administered to the rats, which had been lightly anesthetized with ether, by intragastric intubation. The duration of anesthesia at this point did not exceed 5 minutes.

Methods

Measurement of Absorption of Fatty Acids—At the end of the observation period, the rats were sacrificed by an intraperitoneal injection of sodium pentobarbital (8 mg. per 100 gm. of body weight). The stomach was removed, and its contents were carefully transferred to a beaker by washing it with 95 per cent ethyl alcohol. The intestinal tract was severed at the ileocecal junction. A glass tube was then inserted into the duodenal end and the lumen washed with 200 cc. of 95 per cent ethyl alcohol. The contents of the stomach and small intestine were combined with the washings and analyzed for C^{14} in the lipide.

The cecum and large intestine were incised and their contents carefully transferred to a beaker by means of alcohol washings. The feces, collected from the time of administration of the labeled fatty acid, were added to the same beaker. The entire sample so collected was analyzed for C^{14} in the lipide.

Extraction of Lipides

Contents of Stomach and Small Intestine—The alcoholic extract was decanted and the residue thoroughly ground in a mortar. The ground residue was then recombined with the alcoholic extract referred to above and sufficient ethyl ether added to the mixture to yield a solvent consisting of 3 parts of alcohol to 1 part of ether. The mixture was heated for 1 hour at 55°. The extract was decanted and the residue reextracted with about 100 cc. of a 3:1 alcohol-ether mixture. The combined extracts were filtered and the residue was washed three times with freshly distilled ethyl ether. The filtrates and washings were concentrated at 55° under reduced pressure to a volume of approximately 1 cc.; the last 20 to 30 cc. were removed in an atmosphere of CO₂. The concentrate was extracted five times with hot petroleum ether. The petroleum ether extracts were combined and made up to a desired volume.

Contents of Cecum and Large Intestine and Feces—The alcohol was decanted, the residue thoroughly ground, and the latter recombined with the alcoholic extract in the manner described above. The entire mixture was acidified and, after sufficient amounts of alcohol and ether had been added to provide approximately 200 cc. of 3:1 alcohol-ether, it was extracted at 55° for 1 hour. The extract was decanted and the residue reextracted with approximately 100 cc. of 3:1 alcohol-ether. The residue resulting from this second extraction was refluxed overnight with ethyl ether in a Soxhlet apparatus. All the extracts were now combined and concentrated, and a petroleum ether extract of the concentrate was prepared as described above.

Lymph—The lymph samples were extracted with 20 volumes of a 3:1 alcohol-ether mixture at 55° for 1 hour. The supernatant was decanted and the solids were reextracted, at the same temperature, with 100 cc. of this alcohol-ether mixture. A petroleum ether extract was prepared as described above.

Liver—The lipides were extracted from this tissue without acidification in the manner described for the contents of the cecum and large intestine and feces.

Determination of Lipide-C¹⁴ in Liver and in Contents of Gastrointestinal Tract—Aliquots of the petroleum ether extracts containing total lipides were mounted on aluminum disks for determination of their C¹⁴ content as described by Entenman *et al.* (7).

Determination of Fatty Acid-C¹⁴ in Lymph—Aliquots of the petroleum ether extracts were brought almost to dryness, and the residue was saponified by alcoholic potassium hydroxide for 1 hour on a steam bath. The hydrolysate was acidified by the addition of 20 per cent aqueous H₂SO₄.

and then exhaustively extracted in a liquid Soxhlet with petroleum ether. Aliquots of this petroleum ether extract were transferred to aluminum disks and their C^{14} content was determined (7).

In order to express the C^{14} values as fatty acid- C^{14} , it was necessary to determine the C^{14} content in cholesterol in fractions isolated as described above. Petroleum ether solutions of lymph lipides were evaporated to dryness in an atmosphere of CO_2 , and the residue was saponified as described above. Sufficient alcohol was added to the saponified lipides to raise the concentration of alcohol in the mixture to 50 per cent. Cholesterol was then removed by exhaustive extraction with petroleum ether. Most of the fatty acids, in the form of their soaps, were removed from the petroleum ether extract by washing three times with normal KOH. The cholesterol was then precipitated with digitonin, and the digitonide was freed of fatty acid contaminants by repeated washings with several solvents. The C^{14} content of the cholesterol digitonide was determined as described by Sere *et al.* (8). In none of the samples tested was C^{14} activity found.

Results

In the early intervals, clot formation interferes with lymph flow. For this reason, the rats were kept under constant observation, and any clots that formed were removed as soon as possible. Clots can be readily removed by insertion of a fine barbed piano wire (0.008 inch in diameter) into the cannula in order to entangle the clot. Mann *et al.* (9) have shown that this clot formation ceases about 18 hours after the establishment of the fistula, a finding that has been confirmed in this laboratory. Therefore, at least 20 hours were allowed to elapse before the administration of the labeled fat. In all the rats used in this study, lymph flow was unobstructed during the period following the administration of the labeled material.

Experiments in Which Thoracic Duct Lymph Was Collected—Ten rats with thoracic duct fistulae were studied (Table I). Four received the labeled palmitic acid in the form of tripalmitin, whereas the other six received it as the free fatty acid. Lymph was collected thereafter for 19 to 24 hours.

The rats were sacrificed 19 to 24 hours after the samples of labeled fatty acid were administered, and at this time nine of the ten rats had absorbed 80 to 95 per cent of the labeled fatty acid. Since negligible amounts of the isotope were found in the contents of the stomach and small intestine, it would appear that ample time had been allowed for absorption from the small intestine.

From 36 to 118 cc. of lymph were collected from the rats after the labeled

TABLE I

Recovery of Enterally Administered Labeled Palmitic Acid in Thoracic Duct Lymph

The palmitic acid used was labeled in the carboxyl position with C^{14} . It was dissolved in 0.5 cc. of corn oil at body temperature and administered by intragastric intubation. Each rat received approximately 4 mg. of labeled palmitic acid containing 1×10^5 counts per minute per mg.

Rat No.	Weight	Total lymph collected				Form of labeled palmitic acid administered	Per cent of administered palmitic acid-C ¹⁴ recovered		Per cent of administered palmitic acid-C ¹⁴ absorbed	Per cent of absorbed palmitic acid-C ¹⁴ recovered	
		Before fat administration		After fat administration			In stomach and small intestine contents as lipide-C ¹⁴	In cecum and large intestine contents and feces as lipide-C ¹⁴		In lymph as fatty acid-C ¹⁴	In liver as lipide-C ¹⁴
		gm.	hrs.	cc.	hrs.						
1	200	52	108	20	43	Tripalmitin	2.3	17.2	80.5	85.7	0.34
2	238	28	14	20	36	"	0.5	51.8	47.7	58.3	0.41
3	185	23	42	23	94	"		7.3*	92.7	70.7	0.19
4	287	24.5	38	24	91	"		9.0*	95.0	73.0	0.19
5	200	53	204	19	98	Palmitic acid	1.4	9.8	88.8	70.0	0.35
6	310	27	28	21	101	" "	0.7	6.7	92.6	76.4	0.41
7	386	27.5	49	21	118	" "	2.3	14.5	83.2	78.5	0.26
8	201	21	58	19	78	" "		13.5*	86.5	69.8	0.23
9	183	23	65	19	96	" "		15.5*	84.5	79.7	0.20
10	260	24	12	21	45	" "	0.2	14.2	85.6	91.8	0.25

* The entire gastrointestinal contents were combined for analysis.

TABLE II

Recovery of Enterally Administered Labeled Palmitic Acid in Intestinal Lymph

The palmitic acid used was labeled in the carboxyl position with C^{14} . It was dissolved in 0.5 cc. of corn oil at body temperature and administered by intragastric intubation. Each rat received approximately 4 mg. of labeled palmitic acid containing 1×10^5 counts per minute per mg.

Rat No.	Weight	Total lymph collected						Form of labeled palmitic acid administered	Per cent of administered palmitic acid-C ¹⁴ recovered		Per cent of administered palmitic acid-C ¹⁴ absorbed	Per cent of absorbed palmitic acid-C ¹⁴ recovered	
		Before fat administration				After fat administration			In stomach and small intestine contents as lipide-C ¹⁴	In cecum and large intestine contents and feces as lipide-C ¹⁴		In lymph as fatty acid-C ¹⁴	In liver as lipide-C ¹⁴
		gm.	hrs.	cc.	hrs.	cc.							
20	260	28	20	24	25	Palmitic acid	0.6	6.6	92.8	83.8	0.17		
21	250	23	40	24	46	" "	0.2	64.5	35.3	83.2	0.39		
22	350	22	15	25	52	" "	0.3	13.9	85.8	68.7	1.1		
23	290	20	5	24	24	" "	3.9	6.1	90.0	74.8	0.53		

fatty acid was administered, or about 2 to 6 cc. per hour. Table I shows the percentages of the absorbed C^{14} recovered as fatty acid- C^{14} in lymph. About 58 per cent was recovered in Rat 2. In the other nine rats, from 70 to 92 per cent of the absorbed palmitic acid- C^{14} appeared in the fatty acids of lymph.

Experiments in Which Intestinal Lymph Was Collected—The results of this experiment are recorded in Table II. Three of the four rats studied absorbed 86 to 93 per cent of the labeled palmitic acid introduced into their stomachs. In these rats, from 69 to 84 per cent of the absorbed palmitic acid- C^{14} was recovered in the fatty acid fraction of intestinal lymph.

Despite the fact that only 35 per cent of the administered labeled fatty acid was absorbed by Rat 21 (Table II), the recovery of fatty acid- C^{14} in its intestinal lymph amounted to 83 per cent of that absorbed.

DISCUSSION

In a study involving as complex a process as the transport of absorbed fat by way of the lymphatic stream, it is possible for the condition of the animal to influence that process. For this reason, we must consider the experimental conditions under which thoracic duct lymph was collected. The rats received an anesthetic during insertion of the cannula, less than half an hour, and again at the time that fatty acid was administered, a procedure requiring at most 5 minutes. Insertion of the cannula necessitated a certain amount of intestinal manipulation, but none of the rats was used earlier than 20 hours after insertion of the cannula, and some were not used until 53 hours afterward. Another factor that needs consideration is the loss of lymph before the introduction of the test fat and during the period of lymph collection. It should be stressed that the rats began to drink saline as early as 6 hours after introduction of the cannula and to eat food within 12 hours, and that they continued to do so throughout the entire period of lymph collection. The almost complete absorption of the administered labeled fatty acid also indicates that the experimental procedures had not seriously interfered with the activity of the small intestine.

As much as 92 per cent of the absorbed fatty acids was recovered from the thoracic duct lymph. There can no longer be any doubt that most of the absorbed fat *can* be transported by way of the lymphatic stream. The percentages of the absorbed palmitic acid- C^{14} recovered in lymph were apparently not influenced by the forms in which the labeled fatty acid was administered; *i.e.*, tripalmitin and free palmitic acid. This finding is not consistent with the partition hypothesis of fat absorption proposed by Frazer (10, 11).

Ever since the original experiments, many reviewers have evinced considerable interest in the path of absorption of the 40 per cent of the ingested

fat that was not recovered from the lymph fistula of Munk and Rosenstein's patient, and the conclusion has often been drawn that 40 per cent of absorbed fat passes directly into portal blood. The incomplete recoveries of absorbed palmitic acid- C^{14} in the lymph of the rats reported here would also appear to argue in favor of a portal pathway for absorbed fatty acids. But the possibility cannot be excluded that all of the fatty acid- C^{14} entered the lymph stream and that, subsequently, a portion of it, namely that not recovered in the collected lymph, reached the systemic circulation by way of lymphatic-venous anastomoses. Although evidence for the existence of such anastomotic channels has been presented (12-14), their rôle in fat transport has not yet been assessed. For this reason, the question whether any of the fatty acid- C^{14} passes directly into portal blood must be held in abeyance.

Of considerable interest in this connection is the finding of C^{14} activity in the liver of rats in which thoracic duct lymph had been diverted by cannulation. The small amounts of C^{14} present in the liver at the time the animals were sacrificed (19 to 24 hours after the administration of the labeled fatty acids) does not detract from the significance of this observation because of the ease with which hepatic tissue oxidizes higher fatty acids. The possibility that the C^{14} reached the liver directly by way of portal blood or by lymphatic-venous anastomosis has been pointed out above. Another possibility is the degradation of the labeled palmitic acid during its passage through the intestinal wall and the subsequent passage of the C^{14} into the circulation.

SUMMARY

1. A study on the transport of absorbed C^{14} -labeled palmitic acid via thoracic duct and intestinal lymph of *unanesthetized* rats is presented.

2. The rats received palmitic acid-1- C^{14} either in the form of tripalmitin or as the free fatty acid. In 19 to 24 hours, twelve of the fourteen rats studied absorbed from 81 to 95 per cent of the administered fatty acid- C^{14} .

3. From 70 to 92 per cent of the absorbed palmitic acid- C^{14} was recovered as fatty acid- C^{14} from *thoracic duct* lymph in nine of the ten rats studied.

4. From 69 to 84 per cent of the absorbed palmitic acid- C^{14} was recovered as fatty acid- C^{14} from *intestinal* lymph of four rats.

5. The question of transport of absorbed fatty acids by routes other than the lymph stream is discussed.

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ALTERATIONS IN THE LEVEL OF MUSCLE PHOSPHO-CREATINE OF GUINEA PIGS PRODUCED BY THE INJECTION OF DIPHTHERIA TOXIN*

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An investigation of the acid-soluble phosphorus compounds in the muscle of guinea pigs injected with diphtheria toxin was undertaken in the hope of finding a clue to the nature of the metabolic disorders which must underlie the manifestations of such a toxemia. Although a good deal has been written about the changes in carbohydrate metabolism produced in animals by *Corynebacterium diphtheriae* or its toxin (1, 2), as yet there has been no demonstration of a primary metabolic defect adequate to explain death. Pappenheimer has collected evidence indicating that the diphtheria toxin is the protein part of bacterial cytochrome *b*, and he has suggested that the primary defect produced by the toxin in the host is inhibition of synthesis of a comparable host enzyme (3-6).

Since weakness is a major symptom of this and other infectious diseases, and since death may be looked upon as a failure either to utilize or produce energy adequately, the high energy bond organic phosphorus compounds seemed a logical place to look for a fundamental metabolic defect leading to death. It is well recognized that peripheral circulatory collapse or shock is a frequent finding in terminal infectious states, and it has been shown by Bollman and Flock (7) that shock decreases the muscle stores of high energy phosphate bonds. Studies of arterial and venous blood oxygen content were, therefore, included so that if a change was found in the energy reserves it might be possible to determine whether this was caused by a failure of oxygen transport to the tissues, or by inability of the tissues to utilize adequately the oxygen transported to them because of a defect such as that postulated by Pappenheimer.

Materials and Methods

Guinea pigs, weighing between 360 and 450 gm., before fasting, and fed a diet of Purina rabbit chow supplemented with daily injections of 20 mg.

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of ascorbic acid for at least 6 days immediately before use, were the experimental animals. Acid-soluble phosphorus compounds in the gastrocnemius muscle were studied in four groups of eight animals each. Uninjected animals served as controls; a second group was injected subcutaneously in the abdominal region with 0.25 ml. of a solution of crude diphtheria toxin¹ in physiological saline, each ml. of which contained 3.7 Lf units. To rule out the possibility that effects observed with crude toxin were the results of impurities, a third group received comparable doses of an 85 per cent pure toxin given us by Dr. A. M. Pappenheimer, Jr., while the fourth group was injected with the same dose of purified toxin neutralized with commercial antitoxin. The dose of toxin was selected to give maximum sublethal toxicity in the 18 to 20 hour fast allowed after injection. The animals which received toxin alone all suffered from mild to severe toxicity as evidenced by weakness, ataxia, ruffling of the fur, and in a few cases coma and death. Only those animals which were still alive were used in these studies. Postmortem examination revealed congested adrenals and congestion and hemorrhage in the gut walls and the abdominal lymph glands. The animals which received the neutralized toxin had no clinical or postmortem evidences of toxicity.

Following injection with toxin the animals were fasted from 18 to 20 hours, but allowed water. Nembutal was used as the anesthetic, under which rectal temperatures were taken and one gastrocnemius muscle was removed for acid-soluble phosphorus fraction analysis. Fractionation was carried out by a modification of the method of Umbreit, Burris, and Stauffer (8) described below. Phosphorus concentration was determined in the fractions by the method of Fiske and Subbarow (9) with an Evelyn colorimeter and a 660 m μ filter.

Two other groups of eight guinea pigs each were submitted to studies of arterial and venous blood oxygen and hemoglobin content. An uninjected group served as the controls, while the experimental group received crude diphtheria toxin as above. Blood was taken under nembutal anesthesia from the aorta and common iliac vein. Hemoglobin was determined by the method of Evelyn (10), blood oxygen content by the method of Roughton and Scholander (11), and oxygen capacity was calculated by the hemoglobin-combining capacity as given by Peters and Van Slyke (12).

Statistical analyses were carried out by the methods of Snedecor (13).

EXPERIMENTAL

Since the main object of this study was to measure changes in the high energy phosphorus compounds of muscle in diphtherial toxemia, it was

¹ Obtained through the kindness of the Lederle Laboratories Division, American Cyanamid Company.

felt that the usual methods of separating the phosphorus fractions by employing barium were unnecessarily complex. Simplifications were made and tested in rats so that our results could be compared with those already in the literature. Stimulated muscles with or without a recovery period, as well as resting muscles, were used in order to test the reliability of the simplified method in a variety of situations.

Fractionation was carried out in the following manner. After careful removal under nembutal anesthesia, the gastrocnemius muscle was immediately frozen between two previously flattened blocks of dry ice. An appropriate amount of tissue was then chipped off and weighed on the chilled pan of a micro torsion balance and then ground in 6 per cent trichloroacetic acid in a glass tissue grinder. Grinding and all subsequent steps except hydrolysis and phosphorus determination were performed in the cold room at 3–5°. Following centrifugation, an aliquot of the supernatant was used for 180 and 7 minute phosphorus determinations (180 minute P and 7 minute P), in the usual way, by hydrolyzing in a boiling water bath with 1 N hydrochloric acid and determining the phosphorus content. The difference between these two values is reported as ester phosphorus (ester P). Another aliquot of the deproteinized extract was neutralized with 0.5 N sodium hydroxide to phenolphthalein and the inorganic phosphorus (inorganic P) was precipitated with 10 per cent calcium chloride. The precipitate was taken up in dilute hydrochloric acid and phosphorus was determined. Orthophosphorus (ortho P), comprising phosphocreatine (PCP) and inorganic P, was determined on an aliquot of the neutralized extract by allowing a 20 minute hydrolysis period at room temperature in the acid molybdate solution. Adenosine triphosphate (ATP) was calculated as the difference between the 7 minute P and the ortho P, PCP as the difference between ortho P and inorganic P, and total high energy bond phosphorus ($\sim$ P) was the sum of ATP and PCP. All results were calculated as mg. of phosphorus per 100 gm. of muscle (wet weight), except in the case of ATP for which the 2 labile phosphorus atoms only are reported.

Results

In several cases, phosphocreatine was determined directly on the supernatant fluid after precipitation of inorganic P as a check on the method by difference. The maximum error was 5.2 per cent. The adequacy of the precipitation of inorganic P by calcium chloride was checked by adding known amounts of inorganic phosphate to neutralized aliquots of tissue extracts containing known amounts of inorganic P. Recoveries ranged from 96.2 to 104 per cent of the added phosphorus.

In Table I are presented the results obtained in three groups of eleven

rats each. The first group was resting animals, the second was stimulated through the sciatic nerve by an electronic stimulator 180 times in 1 minute with a 100 gm. weight attached to the severed Achilles tendon and allowed

TABLE I

Results of Phosphorus Determinations on Eleven Rats As Mg. of P Per 100 Gm. of Tissue

Means and standard errors are reported.

Fraction	Resting rats	Stimulated rats, 5 min. recovery	Stimulated rats, no recovery
180 min. P	156.3 $\pm$ 3.4	146.7 $\pm$ 2.6	146.7 $\pm$ 2.4
Ester P	24.3 $\pm$ 1.9	24.3 $\pm$ 1.1	25.2 $\pm$ 1.8
ATP*	41.1 $\pm$ 2.7	37.9 $\pm$ 1.2	38.4 $\pm$ 1.5
PCP	60.3 $\pm$ 1.7	60.0 $\pm$ 2.7	20.0 $\pm$ 1.3
Inorganic P	30.6 $\pm$ 1.9	24.4 $\pm$ 2.9	63.2 $\pm$ 1.3
~P	101.4 $\pm$ 2.2	97.9 $\pm$ 3.1	58.4 $\pm$ 1.9

* The two high energy bond P's only are reported.

TABLE II

Acid-Soluble Phosphorus Fractions in Gastrocnemius Muscle of Guinea Pigs Receiving Purified and Crude Diphtheria Toxin and Purified Toxin Plus Antitoxin

Probability of variations occurring by chance (*P* values) recorded between values to which they apply.

Fraction	Means and standard errors							
	Controls	<i>Vs.</i> <i>P</i> values	Crude toxin	<i>Vs.</i> <i>P</i> values	Pure toxin	<i>Vs.</i> <i>P</i> values	Pure toxin and antitoxin	<i>Vs.</i> controls, <i>P</i> values
180 min.	137.2 $\pm$ 4.8	>0.5	133.5 $\pm$ 4.5	>0.5	135.4 $\pm$ 2.1	0.1-0.05	149.6 $\pm$ 7.6	0.2-0.1
Ester	18.8 $\pm$ 1.8	>0.5	18.8 $\pm$ 1.8	0.5-0.4	20.5 $\pm$ 1.1	0.3-0.2	23.1 $\pm$ 1.9	0.2-0.1
ATP	34.2 $\pm$ 2.0	>0.5	32.8 $\pm$ 1.2	>0.5	33.9 $\pm$ 1.9	0.5-0.4	31.2 $\pm$ 2.8	0.4-0.3
PCP	58.1 $\pm$ 1.2	<0.01	34.8 $\pm$ 2.0	>0.5	34.1 $\pm$ 2.7	<0.01	65.8 $\pm$ 3.7	0.1-0.05
Inorganic P	26.1 $\pm$ 2.1	<0.01	47.2 $\pm$ 2.8	>0.5	46.9 $\pm$ 3.6	<0.01	29.5 $\pm$ 2.9	0.4-0.3
~P	92.3 $\pm$ 2.1	<0.01	67.6 $\pm$ 2.7	>0.5	68.0 $\pm$ 2.9	<0.01	97.0 $\pm$ 4.7	0.4-0.3

a 5 minute recovery period before removal of the muscle, while the third was similarly stimulated but allowed no recovery. There is, in general, good agreement between our results and the results of other investigators

on resting rat and guinea pig muscle, and on stimulated rat muscle with and without a recovery period (7, 14-17). The simplifications are therefore justified for this study, since the method gives reliable results both in resting and stimulated animals.

The results of the phosphorus determinations in animals injected with diphtheria toxin are shown in Table II. It will be seen that both crude and pure diphtheria toxin produced almost identical statistically significant decreases in PCP, with a comparable rise in inorganic P. A comparable fall in $\sim$ P took place since the ATP values were unchanged. There were no significant differences in the results produced by the pure and crude

TABLE III

Effect of Subcutaneous Injection of Crude Diphtheria Toxin on Arterial and Venous Blood Oxygen Content of Seven Guinea Pigs

The blood was taken from the aorta and the common iliac vein or inferior vena cava just above the junction of the common iliac veins. Means and standard errors are reported.

Blood components		Control animals	Experimental animals	P values
Arterial blood	O ₂ content, vol. %	15.5 $\pm$ 0.5	21.8 $\pm$ 0.8	<0.01
	Hemoglobin, gm. %	12.5 $\pm$ 0.3	19.0 $\pm$ 0.5	<0.01
	O ₂ saturation, %	92.6 $\pm$ 1.4	86.0 $\pm$ 3.0	0.1-0.05
Venous blood	O ₂ content, vol. %	10.9 $\pm$ 0.5	7.1 $\pm$ 0.7	<0.01
	Hemoglobin, gm. %	12.5 $\pm$ 0.3	18.4 $\pm$ 0.5	<0.01
	O ₂ saturation, %	65.1 $\pm$ 2.8	28.9 $\pm$ 3.0	<0.01
Oxygen arteriovenous difference	Vol. %	4.6 $\pm$ 0.5	14.7 $\pm$ 0.8	<0.01

toxins, and injection of pure toxin neutralized with antitoxin caused no significant changes from the normal values.

The results of the blood oxygen studies are reported in Table III. Injection of crude diphtheria toxin produced a marked hemoconcentration with an associated marked increase in arterial oxygen content, as there was only a slight decrease from the control value in the arterial oxygen saturation. On the venous side there was a marked fall in oxygen saturation, although the oxygen content was only slightly below the normal level. The arteriovenous oxygen difference rose markedly following the injection of toxin.

Rectal temperatures in the animals which received unneutralized toxin ranged from 26.7-37.8°, while the controls varied from 34.8-38.8°. The marked fall below the normal range in some of the controls is probably the result of anesthesia (18).

DISCUSSION

The fall in PCP and consequently in $\sim P$ represents a significant lowering of the muscle energy reserves. The fact that the crude and pure toxin produced the same change and that this could be prevented by neutralization of the toxin with antitoxin before injection indicates that this effect was the result of the toxin itself and not of some impurity carried over from the culture medium.

Since the high energy phosphorus compounds are the only known source of energy for muscular and chemical work in the body, a reduction in the reserve of these compounds is obviously a serious metabolic defect. This is particularly true in resting muscle in which the rate of utilization of ATP and PCP is at a minimum, and consequently implies a much larger percentage decrease in the maximum rate of synthesis of $\sim P$ than the percentage fall in $\sim P$ content. The significance of this change is enhanced by the fact that it occurred, in most of the animals at least, before they were moribund, and several hours before they would be expected to die. Thus the phenomenon is not merely a terminal one, and may have some causal relationship to the manifestations of toxemia and ultimately to death from the toxemia.

The arteriovenous oxygen differences might at first glance suggest that the fall in $\sim P$ may be the result of shock, since a high arteriovenous oxygen difference, which the toxemic animals exhibited, is one of the characteristics of shock (19), and since it is known that shock causes a fall in muscle $\sim P$ (7). This latter finding is to be expected because shock is essentially a failure of oxygen transportation to the tissues, and because the synthesis of high energy bond phosphate is predominantly an aerobic process. The real question is then whether or not these figures represent a failure of oxygen transportation to the tissues with consequent tissue anoxia.

The adequacy of oxygen supply to the tissues depends on the relative rates of tissue utilization and blood transportation of oxygen. There is no evidence of an increased rate of tissue utilization of oxygen in the experimental animals. In fact quite the reverse is true, since the muscles are at rest and there is a fall in body temperature at this stage of the toxemia. The fact that the venous content of oxygen is nearly normal indicates that more oxygen is being transported to the tissues than they need, since in tissue anoxia caused by shock the venous blood draining the leg has a markedly lowered oxygen content frequently approaching zero (20-22). It is conceivable that tissue anoxia might have existed in these animals in the presence of considerable amounts of venous oxygen if the rate of blood flow through the capillaries was too rapid to allow adequate

time for diffusion of oxygen into the tissues. In the animals receiving diphtheria toxin the circulation was slowed rather than speeded up, however, as shown by the fact that the veins were narrower than normal and that blood could be withdrawn from the aorta or common iliac veins only at a much slower rate than in the controls.

It would seem likely, therefore, that defective oxygen transportation and tissue anoxia were not the cause of lowered muscle $\sim$ P reserves, and that the explanation must be sought in either defective tissue utilization of oxygen because of some such defect as that postulated by Pappenheimer, or in defective coupling of inorganic phosphorus uptake to oxidation.

SUMMARY

Diphtheria toxin, both crude and purified, when injected into guinea pigs, produced a fall in phosphocreatine and, since adenosine triphosphate remained unchanged, a comparable fall in total high energy phosphates in the gastrocnemius muscle. Toxin neutralized with antitoxin failed to produce these changes. Toxin also produced a marked hemoconcentration and increase in the arteriovenous oxygen difference in the blood supplying the hind leg. The oxygen content of the venous blood was only slightly reduced, however. The causal relationships between the changes in circulatory status and the acid-soluble phosphorus compounds, as well as the relationship between these findings and the manifestations of toxemia, are discussed.

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ENZYMATIC DEAMINATION OF CYTOSINE NUCLEOSIDES*

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The existence of enzymes which deaminate purine and pyrimidine compounds has been known for decades. Nevertheless, the only enzymes which have been well characterized and purified are those which deaminate purine compounds (see (1-3)). The deamination of cytosine and its derivatives has been observed in various tissues and microorganisms. Schmidt (1) found that a crude extract of rabbit liver deaminated guanine, guanosine, adenosine, and cytidine. On fractionation he obtained a purified adenosine deaminase, which had no activity on cytidine, and concluded that a separate cytidine deaminase must have been present in the crude extract. When ribose nucleic acids are treated with autolysates of calf intestinal mucosa, the products isolated are inosine, guanosine, and uridine. It is known that adenosine deaminase is present and is responsible for the formation of inosine. Since the pyrimidine nucleoside fraction is almost entirely uridine, it is concluded that a cytidine deaminase is present, but its identity has not been established. This enzyme in intestinal mucosa apparently does not convert cytosine desoxyriboside to the corresponding uracil nucleoside (Klein (3)). Conway and Cooke have reported the production of ammonia from cytidine by fowl's blood but not by mammalian blood (4). Greenstein *et al.* (5) tested a large variety of tissues from rats and mice and observed deamination of cytidine only with mouse kidney, whereas most of the tissues tested deaminated adenosine and guanosine.

It appears certain, therefore, that a cytidine deaminase exists, although perhaps with a limited distribution. To the best of our knowledge this enzyme has never been obtained free of the other nucleo-deaminases. We have found cytidine deaminase in brewers' yeast and in *Escherichia coli*. An extract can be prepared from the latter organism which deaminates only cytosine riboside and desoxyriboside. This provides a tool for the

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quantitative determination of these compounds.¹ For activity measurements use is made of the fact that the spectra of cytosine and uracil differ sufficiently to permit the use of spectrophotometric techniques to follow the deamination reaction.²

Materials

The compounds used in this study had the following origins: adenine and cytidylic acid, Nutritional Biochemicals Corporation; adenosine, guanine, guanosine, uracil, uridine, Schwarz Laboratories, Inc.; cytosine and isocytosine, Dougherty Chemicals; cytidine, prepared from cytidine nitrate kindly furnished by Dr. Gerhard Schmidt; cytosine desoxyriboside, isolated by Dr. L. A. Manson in this laboratory by the method of Klein (3). Although the configuration of the desoxypentose moiety has not yet been established, the name of this nucleoside is retained in accordance with previous usage. Tris(hydroxymethyl)aminomethane was generously supplied by the Commercial Solvents Corporation. The technical product received was treated in aqueous solution with activated charcoal, then recrystallized twice from ethanol. The final product was in the form of glistening large needles, m.p. 169–170° (uncorrected); aqueous solution colorless. This was used for the preparation of buffer solutions according to Gomori (6). *E. coli* strain 15 (9723 of the American Type Culture Collection) was used as the source of the enzyme in most of this work.³ Brewers' yeast was the gift of Anheuser-Busch, Inc., of St. Louis. Alumina powder A303 was generously supplied by the Aluminum Company of America.

EXPERIMENTAL

Extract of intestinal mucosa was prepared by the method of Klein (3). The extract was approximately 1 year old at the time it was used. Dried *brewers' yeast* was autolyzed according to Lebedev (7). The crude yeast juice and also the fraction precipitated between 0.45 and 0.65 saturation with ammonium sulfate (a solution of ammonium sulfate saturated at room temperature and adjusted to pH 7.5 with ammonium hydroxide was added) were used in the optical test. Stock cultures of *E. coli* were kept on agar slants of Medium 1 and were transferred monthly. All incubations were at 37°. Medium 1 contained 0.3 per cent beef extract (Difco), 0.7 per cent peptone (Difco), 0.2 per cent yeast extract (Difco), and 0.4 per cent glucose. Medium 2 contained 0.4 per cent beef extract, 0.8 per cent peptone, and 0.4 per cent tryptose (Difco). The desired volume of

¹ Sable, H. Z., Lampen, J. O., and Wang, T. P., in preparation.

² While this work was in progress, Ploeser and Loring (9) published ultraviolet absorption curves which are in essential agreement with ours.

³ We have also observed a potent cytidine deaminase in extracts of strain E26.

medium was inoculated with 1 per cent by volume of a 24 hour subculture of the organism in liquid Medium 1. After 24 hours' incubation the cells were collected by centrifugation, washed twice with 0.9 per cent NaCl or 1 per cent KCl, and ground in an ice-cold mortar with alumina powder for 5 minutes (8). The paste was then mixed with 10 to 20 volumes of cold 0.1 M phosphate buffer, pH 7.6 to 7.7, allowed to stand for 30 minutes at 2°, and centrifuged at $20,000 \times g$ for 10 minutes in a Servall centrifuge.

Extracts were also prepared from cells dried overnight in a vacuum desiccator over CaCl_2 . About 35 mg. of the dried cells were triturated with 5 ml. of the phosphate buffer and the extract clarified by centrifugation. To obtain acetone-dried cells, 0.35 gm. of wet cells was suspended in 5 ml. of the phosphate buffer and the suspension treated with 25 ml. of acetone (-20°). The cells were collected by centrifugation, washed with 25 ml. of cold acetone, and dried in a vacuum desiccator. The dried cells were extracted with buffer as above.

Determination of Deaminase Activity—Detailed spectra for cytidine and uridine are given by Ploeser and Loring (9) and will not be presented here. The absorption curve of cytidine in neutral solution is similar to Curve A (Fig. 1) and that of uridine to Curve B. The changes in extinction at several wave-lengths accompanying the deamination of cytidine to uridine are given in Table I. The values of ϵ^4 obtained for cytidine at 270 $m\mu$ and for uridine at 262 $m\mu$ are in agreement within experimental error with those of Ploeser and Loring. At 230 $m\mu$ many materials have high "end absorption," and most purines have high absorption near 256 $m\mu$. We have therefore selected 282 $m\mu$ as the most suitable wave-length for the spectrophotometric test. The general procedure was as follows: a suitable amount of extract was added to the substrate solution buffered with tris(hydroxymethyl)aminomethane (pH 7.8, final concentration 0.025 M) in a 1 cm. cuvette. The final volume in each case was 3.0 ml. The decrease of extinction at 282 $m\mu$ was measured with a Beckman spectrophotometer, model DUV. Readings were taken as soon as possible after addition of the extract and then at intervals of 30 seconds for at least 5 minutes if a rate was to be measured, or until the end of the reaction if complete deamination was desired. The readings were plotted against time as abscissa, and extrapolated to zero time. Any variations from this procedure are described with the individual experiments.

The spectrum of cytosine desoxyribose is very similar to that of cytidine.⁵ We have assumed that the spectrum of uracil desoxyribose is identical with that of uridine. Certainly the changes in extinction which

⁴ ϵ_λ is the molecular extinction coefficient = $(1/c)(1/d) \log_{10}(I_0/I)$, where c is expressed in moles per liter and d in cm. $E_\lambda = \log_{10}(I_0/I)$. I_0 and I are the incident and emergent light intensities respectively.

⁵ Manson, L. A., and Lampen, J. O., unpublished results.

CYTOSINE NUCLEOSIDE DEAMINATION

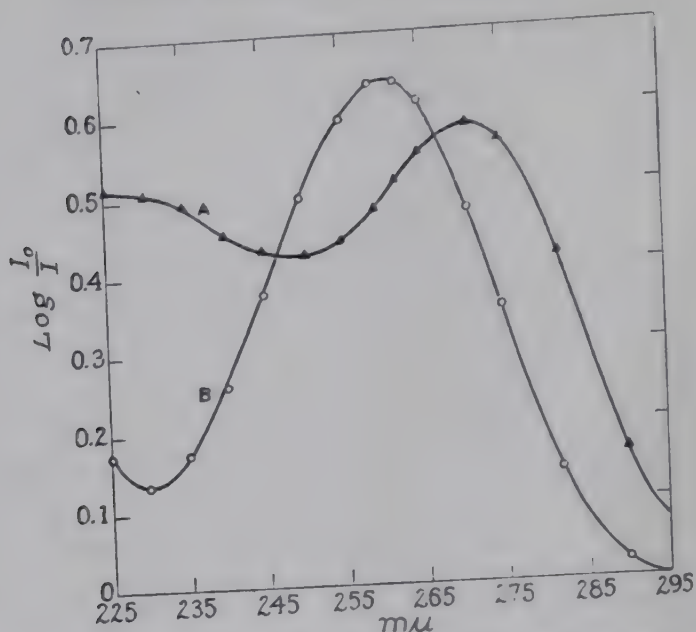


FIG. 1. Spectral changes during the deamination of cytidine by an extract of *E. coli* cells. A mixture of 2.0 ml. of a cell-free extract (equivalent to 94 mg. of wet cells), 8.0 ml. of tris(hydroxymethyl)aminomethane buffer (0.05 M, pH 6.9), and 49 μ M of cytidine was incubated at 38°. Samples were deproteinized with an equal volume of 4 per cent perchloric acid, and the filtrates adjusted to pH 4.5 with 4 N sodium acetate. A 0.2 ml. aliquot of this solution was diluted to 3.0 ml. with 0.1 M phosphate buffer (pH 7.6) and the absorption spectrum determined with the Beckman spectrophotometer against a control incubation without cytidine. Curve A, before incubation; Curve B, after 90 minutes incubation. The value for ammonia formation is given in Experiment B, Table III.

TABLE I

Changes in Molecular Extinction Coefficients Accompanying Deamination of Cytidine to Uridine

Neutral aqueous solutions of the nucleosides were used.

Wave-length	Cytidine, $\epsilon \times 10^{-4}$	Uridine, $\epsilon \times 10^{-4}$	Change during cytidine deamination, $\Delta\epsilon \times 10^{-4}$
<i>mμ</i>			
230	0.78	0.22	-0.56
256	0.66	0.94	+0.28
262		1.00*	
269.5	0.87(-)	0.86(+)	<-0.01
270	0.87*		
282	0.60	0.27	-0.33

* Absorption maxima.

accompany deamination appear to be the same for the two cytosine nucleosides (see below). We have, therefore, accepted ΔE_{282} as a criterion of deamination of the desoxyriboside as well. Quantitative treatment of

this deamination must, however, await the preparation of uracil desoxyribose and the determination of its spectrum. This is in progress.

The action of the enzyme preparations on other purine and pyrimidine compounds was studied at wave-lengths at which a change in extinction was to be expected if a reaction occurred. The wave-lengths used are given with the actual data.

Experiments with Calf Intestinal Mucosa and with Lebedev Juice—The year-old autolysate of calf intestinal mucosa was tested for its ability to deaminate adenosine. With 0.10 ml. of extract (equivalent to 10 mg. of wet tissue) and 0.140 μM of adenosine, a rate of $\Delta E_{265} = -0.050$ per minute was observed. With the same amount of extract and 0.305 μM of cytidine there was no change in absorption at 282 $\text{m}\mu$. In view of Klein's indirect evidence that there is a cytidine deaminase in this tissue, it would be of interest to test a fresh autolysate.

Lebedev juice was used in a similar experiment. The test cuvettes contained 0.02 ml. of enzyme solution (equivalent to 6.6 mg. of dry yeast) and 0.140 μM of adenosine or 0.305 μM of cytidine. With adenosine there was no change in extinction at 265 $\text{m}\mu$. With cytidine the ΔE_{282} observed corresponded to 36 per cent deamination in 44 minutes. From these results it is clear that adenosine deaminase and cytidine deaminase are separate enzymes. This confirms the findings of Schmidt (1) on rabbit liver.

An attempt was made to purify the enzyme present in autolyzed yeast juice. Fractionation was carried out with ammonium sulfate at pH 7.5. The fraction precipitated between 0.45 and 0.65 saturation contained most of the activity. Further fractionation was attempted by isoelectric precipitation at pH 4.7. However, this treatment appeared to destroy the enzyme. Experiments with yeast were abandoned when *E. coli* was found to be a richer source of the deaminase.

Experiments with Cell-Free Extracts of E. coli—The results obtained with alumina extracts, as well as with extracts of vacuum-dried or acetone-dried cells, are given in Table II. The enzyme can be extracted from the alumina-ground or vacuum-dried cells; however, the yield of enzyme is better in the former case. The extract of acetone-dried cells was inactive. Whether the enzyme was inactivated or simply not extracted is not known. That acetone may have a destructive effect was shown by the following experiment. The proteins of an extract of alumina-ground cells were precipitated by cold acetone and the resulting powder extracted with the phosphate buffer. The solution was inactive in the deamination of cytidine.

The yield of deaminase either per unit weight of cells or per unit weight of protein in the extracts was definitely lower in the presence of glucose and yeast extract (Medium 1) than in their absence. The total yield of en-

zyme is much greater, however, with Medium 1, since the cell yield is over 3 times that from Medium 2. For this reason an extract of cells from Medium 1 was used in all subsequent experiments.

TABLE II

Cytidine Deamination by Various Cell-Free Extracts of E. coli

Each cuvette contained 0.114 μM of cytidine and 0.1 ml. of the extract.

Extract	Medium No.	Weight of wet cells corresponding to 1 ml. extract	Protein* in extract	Per cent deamination	
				4 min.	15 min.
		mg.	mg. per ml.		
Alumina.....	2	56	4.7	62	99
“ frozen and thawed.....	2	56	4.7	50	99
“	1	87	7.2	49	99
“ after dialysis	1	87	5.4	28	92†
Vacuum-dried cells.....	1	70	3.9	23	103‡
Acetone-dried “	1	70		0	

* Protein determined by the method of Robinson and Hogden (10).

† 21 minutes.

‡ 32 minutes.

TABLE III

Ammonia Production during Deamination of Cytosine Nucleosides

The final volume of the reaction mixtures was 10 ml., the pH was 6.90, and the temperature of incubation 38°. The final concentration of tris(hydroxymethyl)-aminomethane buffer was 0.048 M in Experiment A and 0.04 M in the others. The incubation time was 120 minutes in Experiment A and 90 minutes in Experiments B and C.

Experiment No.	Substrate	Amount	Enzyme	NH ₃ formed*
		μM	ml.	μM
A	Cytidine	19.3	0.5†	20.8
B	“	49.0	2.0‡	48.6
C	Cytosine desoxyriboside	54.6	2.0‡	59.6

* The NH₃ formed was aerated into standard acid. In Experiment A it was nesslerized (11); in Experiments B and C it was estimated by titration of the excess acid.

† Equivalent to 44 mg. of wet cells of *E. coli*

‡ Equivalent to 94 mg. of wet cells of *E. coli*.

There does not appear to be an essential dialyzable cofactor for the deamination. A 2 ml. portion of the extract was dialyzed for 24 hours at 2° against two 10 liter portions of distilled water. The dilution which occurred during dialysis was calculated from the data of Table II on the

protein concentration of these extracts. The results show that about 75 per cent of the original activity was retained.

Freezing the extract did not inactivate the enzyme. Preparations kept 4 months at -20° retained their original activity.

Nature of Reaction—Two types of evidence indicate that the action of the cell-free extracts on cytosine nucleosides is a hydrolytic deamination: (1) the spectral changes correspond to a quantitative conversion of cytidine to uridine (Fig. 1), and (2) approximately 1 mole of ammonia is pro-

TABLE IV

Action of Extract of E. coli on Purine and Pyrimidine Derivatives

Each cuvette contained 0.1 ml. of extract equivalent to 5 to 10 mg. of wet cells.

Compounds	Concentration	Wave-length	Time	ΔE observed
	$M \times 10^5$	$m\mu$	min.	
Adenine.....	5.71	265	10	0
Adenosine.....	4.67	265	13	0
Cytosine.....	8.36	282	14	0
Isocytosine.....	26.4	282	8	0
Cytidine.....	10.6	282	21	-0.354*
Cytosine desoxyriboside.....	12.1	282	14	-0.371
Cytidylic acid.....	4.30	282	17	0
“ “ + phosphatase†.....	4.30	282	16	-0.126‡
Guanine.....	5.16	248	10	0
Guanosine.....	3.35	255	5	0
Uracil.....	9.01	282	14	0
Uridine.....	7.72	282	15	0

* Theoretical $\Delta E_{282} = -0.355$.

† A purified alkaline phosphatase preparation generously provided by Dr. G. Schmidt.

‡ Theoretical $\Delta E_{282} = -0.144$.

duced per mole of nucleoside (Table III). We therefore represent the reactions by the following equations:



Fig. 1 shows the spectral changes that occur during the action of the extract on cytidine. Curve B, obtained after incubation of the mixture, corresponds closely to that of uridine; however, the extinction at $262 m\mu$ is lower than would be expected for a complete conversion of the original cytidine to uridine. This extract contained a potent pyrimidine nucleoside phosphorylase.⁶ Also the concentration of phosphate in this experi-

⁶ Manson, L. A., Wang, T. P., and Lampen, J. O., unpublished results.

ment (0.02 M) was significantly higher than in the usual activity test. It is probable, therefore, that the low extinction observed after the deamination is the result of subsequent phosphorolysis of a portion of the uridine to yield uracil, which has a lower extinction at 262 m μ . The formation of uracil has been confirmed by utilizing the fact that in alkaline solution uracil has a significant absorption at 300 m μ , while uridine has not (Hotchkiss (12)).

Similar spectral changes have also been obtained when cytosine desoxy-ribose was the substrate (Experiment C, Table III). The absorption curves are not shown here, since they can be superimposed almost exactly on those of Fig. 1 for the cytidine experiment.

Specificity of Enzyme—Eleven purine and pyrimidine compounds were tested (Table IV). Only the cytosine nucleosides were attacked. When

TABLE V

Effect of Enzyme Concentration on Rate of Cytidine Deamination

Each cuvette contained 0.111 μ M of cytidine. The theoretical ΔE_{262} for complete deamination of the cytidine is -0.123.

Enzyme*	Initial rate, ΔE_{262} per min.	Total observed ΔE_{262}
ml.		
0.05	-0.009	-0.124
0.10	-0.015	-0.120
0.20	-0.035	-0.133

* The extract contained 7.2 mg. of protein (equivalent to 87 mg. of wet cells) per ml.

phosphatase is added, cytidylic acid is also attacked, presumably through its prior conversion to cytidine and subsequent deamination. The absence of the other deaminases is unexpected in view of the previously reported occurrence of adenosine deaminase (13) and cytosine deaminase (14) in other strains of *E. coli*. The formation of certain deaminases is inhibited by the presence of glucose (15), but we have observed that extracts from cells grown in the absence of glucose and yeast extract (Medium 2) are also inactive on adenosine and cytosine. Thus an inhibition by glucose does not appear to be involved here.

Characteristics of Deamination Reaction—The data of Table V demonstrate a good proportionality between the concentration of enzyme and the rate of deamination. The total change in extinction is close to the theoretical value in each instance. Similar results have been observed in extracts of *E. coli* cells grown on Medium 2.

Preliminary studies of the pH optimum of the enzyme have shown a rate of deamination about 50 per cent higher in phosphate buffers (pH 5.9 to

7.35, 0.05 M) than in tris(hydroxymethyl)aminomethane buffers (pH 7.25 to 8.7). Purification of the enzyme and especially removal of the nucleoside phosphorylases will probably be necessary before one can determine the

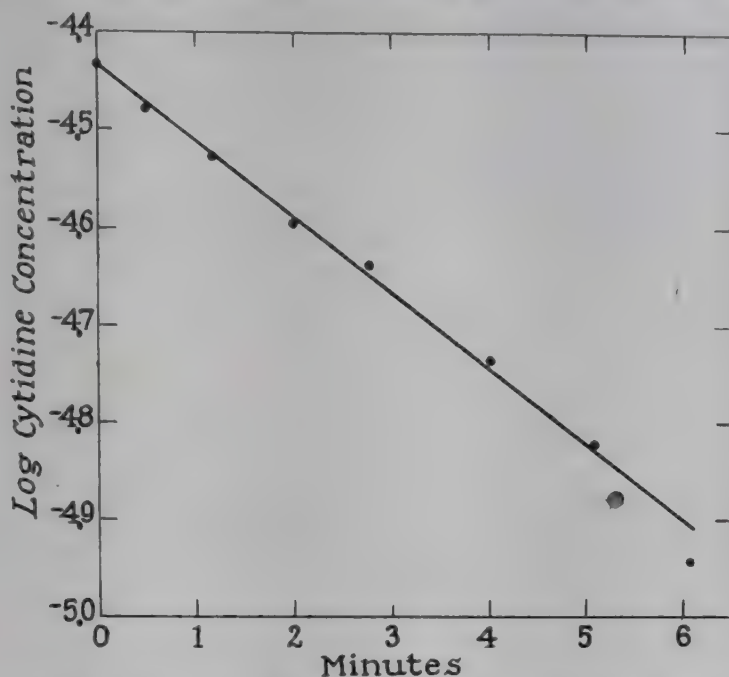


FIG. 2. Kinetics of cytidine deamination. Each cuvette contained 0.1 ml. of extract (equivalent to 8.7 mg. of wet cells) and $0.111 \mu\text{M}$ of cytidine. See the text for details of the calculation of the cytidine concentration. The concentration is expressed in moles per liter.

TABLE VI

Rate of Deamination of Cytidine and Cytosine Desoxyriboside by Extracts of *E. coli*

Extract	Substrate	Concentration	Time*	ΔE_{232}	Initial rate, ΔE_{232} per min.
		$\mu\text{M per ml.}$	min.		
A†	Cytidine	0.106	5	-0.106	-0.021
	Cytosine desoxyriboside	0.121	2	-0.164	-0.082
B‡	Cytidine	0.483	5	-0.103	-0.021
	Cytosine desoxyriboside	0.420	3	-0.178	-0.059

* The time intervals selected were such that all measurements were made on the linear portion of the deamination curve.

† 0.1 ml. (equivalent to 4.7 mg. of wet cells) was used.

‡ 0.11 ml. (equivalent to 1.1 mg. of wet cells) was used.

mechanism of this effect. The present data indicate, however, that the deaminase has a broad pH optimum.

The deamination of cytidine in aqueous solution follows first order kinetics over at least a major portion of its course (Fig. 2). In this experi-

ment the concentration of cytidine was obtained in the following manner: the ΔE_{232} in a particular experiment represents the conversion of cytidine to uridine at various times and is a function of the uridine formed. Therefore the concentration of cytidine remaining is a function of the difference between the theoretical ΔE (ΔE calculated from the amount of cytidine added) and the observed ΔE at the particular time concerned.

The first order kinetics obtained here are in agreement with the mechanism proposed earlier for this reaction. A hydrolytic deamination in aqueous solution should follow a pseudomonomolecular rate law.

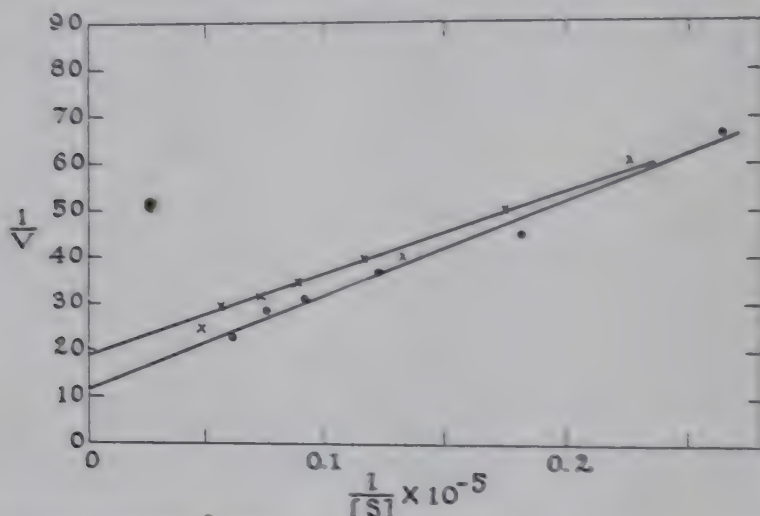


FIG. 3. Relation between substrate concentration and rate of deamination. Each cuvette contained 2.5 ml. of 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 7.8), 0.1 ml. of extract, and the indicated quantity of substrate in a total volume of 3.0 ml. The velocity is expressed as ΔE_{232} per minute and substrate concentration in moles per liter. Cytidine (●), cytosine desoxyriboside (X). The volume of extract used was equivalent to 8.7 mg. of wet cells in the experiment with cytidine and to 1.5 mg. in the experiment with cytosine desoxyriboside.

Relative Rate of Deamination of Cytosine Nucleosides—Extracts of *E. coli* cells deaminate cytosine desoxyriboside about 3 to 4 times as rapidly as cytidine in equivalent concentration. Table VI shows representative experiments with two extracts.

Since the cytidine used was prepared by a method involving heavy metal precipitation, it was thought possible that the slower deamination of cytidine was the result of inhibition of the enzyme by heavy metal impurities present in the cytidine. In another experiment H_2S was passed for 40 minutes through the cytidine solution used in Experiment A of Table VI. After filtering twice through Whatman No. 5 filter paper and aerating until no H_2S remained, the cytidine solution was tested again. The rate of deamination was identical with that obtained before treatment with H_2S .

Therefore, the slow deamination of cytidine is apparently not due to contamination by heavy metals.

Determination of Michaelis Constants for Cytosine Nucleosides—Since the rates of deamination of cytidine and cytosine desoxyriboside are different, it is of interest to know whether or not the dissociation constants of their respective enzyme-substrate complexes differ as well. The Michaelis constants for the two complexes were determined by the method of Lineweaver and Burk (16). The data are plotted in Fig. 3. The lines were fitted to the experimental points by the method of least squares (17). The equations for the lines are as follows:

$$\text{For cytidine, } \frac{1}{v} = 11.65 + 202.0 \frac{1}{S}$$

$$\text{For cytosine desoxyriboside, } \frac{1}{v} = 19.10 + 170.8 \frac{1}{S}$$

where v is the initial reaction rate and S is the substrate concentration. The K_m values obtained from these equations are 1.74×10^{-4} M for cytidine and 8.9×10^{-5} M for cytosine desoxyriboside.

The authors are indebted to Professor C. F. Cori for his interest and suggestions during the course of this investigation.

SUMMARY

1. Cytidine and cytosine desoxyriboside were deaminated by extracts of cells of a particular strain of *Escherichia coli*, the latter 3 to 4 times as rapidly as the former. The rate of deamination was followed by measuring the decrease in absorption at 282 m μ in the Beckman spectrophotometer. The final spectral change ($\Delta\epsilon_{282} = -3300$) corresponded to the quantitative conversion of cytidine to uridine, and there was 1 mole of ammonia formed per mole of nucleoside. This indicates that the reaction is a hydrolytic deamination.

2. First order kinetics were observed for the major part of the reaction. The Michaelis constants were 1.74×10^{-4} M for cytidine and 8.9×10^{-5} M for cytosine desoxyriboside. The enzyme solution was not inactivated by prolonged dialysis or by freezing. Acetone treatment of the enzyme solution caused inactivation.

3. Adenine, adenosine, cytosine, isocytosine, guanine, and guanosine were not deaminated by these cell-free extracts. Cytidylic acid was deaminated only when intestinal phosphatase was added to the extracts.

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PURIFICATION OF LIVER ESTERASE*

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During the course of other investigations in this laboratory, it became necessary to employ an esterase preparation of greater purity than had been reported in the literature. Baker and King (1) had been able to purify a liver esterase some 12-fold over a powder extract by a series of precipitations with acetic acid and sodium sulfate. Bamann and Laeverenz (2) prepared what was thought to be a crystalline pancreatic esterase, but Boissonnas (3) was unable to confirm this work. The latter author obtained a pancreatic enzyme of only some 17-fold increase in purity which still exhibited eight components in the electrophoretic field.

Toward the completion of our study, Mohammed (4) described the crystallization of esterase from horse liver acetone powder and found the enzyme preparation to be homogeneous in the electrophoretic field. However, when we tested an approximately 70-fold purified esterase using Mohammed's assay for esterase, this preparation appeared to have nearly twice the activity reported for the crystalline material. Furthermore, upon attempting to prepare crystalline esterase according to Mohammed, a material was obtained, which, although crystalline, was very low in esterase activity. Even this low activity disappeared on recrystallization.¹ Hence it seemed necessary to proceed with our purification procedure.

Our best esterase preparation is a clear colorless solution 270 times purer than the horse liver acetone powder used as starting material. We have repeated the preparation several times with comparable results.

EXPERIMENTAL

Esterase Assay—A manometric assay was employed. It was based on the liberation of carbon dioxide from bicarbonate by the butyric acid formed from enzymatic hydrolysis of methyl butyrate. The assay was carried

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¹ It is of interest that the recrystallized product possessed considerable alcohol dehydrogenase activity, and in view of the fact that the method of preparation is very similar to that of Bonnicksen and Wassén (5) for crystalline alcohol dehydrogenase, it is possible that these products are identical.

out in Warburg flasks in an N_2 - CO_2 atmosphere at pH 7.4 and with a final volume of 3.3 ml. The main compartments contained 0.45 ml. of 0.15 M $NaHCO_3$, 0.038 ml. of 100 per cent methyl butyrate (final concentration 0.10 M), and distilled water to make a final volume of 2.70 ml. The side arms contained 0.10 ml. of 0.15 M $NaHCO_3$, from 0.10 to 0.40 ml. of properly diluted enzyme, and distilled water to make a final volume of 0.60 ml. A gas mixture containing 95 per cent N_2 -5 per cent CO_2 was passed through the vessels for 4 minutes, the stop-cocks closed, and temperature equilibrium (30°) established. After dumping the contents of the side arm, readings were taken at 4 minute intervals for 30 minutes, when approximately 150 to 200 μ l. of CO_2 were liberated.

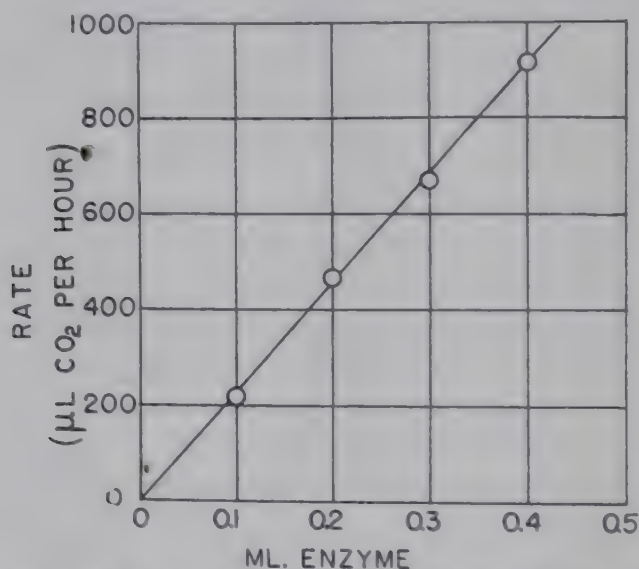


FIG. 1. Proportionality between enzyme concentration and rate of reaction in manometric assay. The esterase was the preparation from Stage VI diluted 1:200. The manometric work is described under "Esterase assay."

Under the conditions employed a zero order reaction was obtained, and the rate of CO_2 evolution was found to be proportional to the amount of enzyme employed. The latter relation is illustrated in Fig. 1 with use of the highly purified esterase, but this relation also held true with cruder preparations. No corrections for CO_2 retention were necessary because of the great dilution of the enzyme preparations assayed by this procedure.

In experiments in which the pH was varied, the colorimetric hydroxamic acid determination for esters described by Hestrin (6) was utilized. In such experiments the incubations were carried out in 15 ml. test-tubes at a volume of 3.30 ml., with borate buffer replacing bicarbonate. When working below pH 7.0, we added 3.3 per cent mannitol to depress the pH of the borate buffer. Other conditions remained the same as in the manometric assay. After the esterase action was stopped by the addition of

0.70 ml. of 4 N HCl, 0.5 ml. aliquots were diluted to 25.0 ml. with distilled water, and 1.0 ml. amounts of the latter solution were used directly for the hydroxamate color reaction.

Enzyme Units and Purity—A unit of esterase was defined as that amount which, under the conditions described above, liberated 1000 μ l. of CO₂ per hour. Dry weights were determined after thorough dialysis against distilled water and drying at 110°. Purity was expressed as units per mg. of dry weight.

Purification of Enzyme

The details of the procedure together with yields and purity follow.

Stage I. Preparation of Acetone Powder—About 6 pounds of fresh horse liver² were obtained from the slaughter-house and ground to a fine mince in an electric grinder. A 2000 gm. portion of this material was thoroughly stirred at room temperature with 8 liters of ice-cold acetone. The mixture was drained through a large Büchner funnel (without paper) and the residual acetone squeezed from the material by hand. Two further washings with 8 liters of acetone were carried out. The acetone-washed material was then spread out in a thin layer on a table top to dry at room temperature for 12 hours. The dry material was ground in a burr mill with care to avoid overheating. Approximately 560 gm. of acetone powder were obtained from the 2000 gm. of liver mince.

The acetone powder was stable for at least 3 months at 5°. 1 gm. of powder contained 1058 units of purity 1.02.

Stage II. Water Extraction—100 gm. of acetone powder were added to 1 liter of cold distilled water and the material extracted by stirring in the cold for 12 hours. At the end of this time the mixture was centrifuged in a refrigerated centrifuge and the supernatant liquid saved. Volume 840 ml., total units 55,430, recovery 53 per cent, purity 3.2.

Stage III. Ammonium Sulfate Fractionation—To the 840 ml. of solution from Stage II was added an equal volume of saturated (at 5°) ammonium sulfate solution neutralized to pH 7.50 with ammonia. The mixture was allowed to stand in the cold for 12 hours and the precipitate was then removed by centrifugation.

For every 100 ml. of supernatant fluid, 10 gm. of solid ammonium sulfate were added with stirring. This mixture was likewise allowed to stand in the cold for 12 hours, after which the precipitate was collected by centrifugation. The precipitate was dissolved in 0.145 M phosphate buffer, pH 7.5, and made up to 130 ml. Total units 42,750, recovery for this step 77 per cent, purity 8.6.

² Beef liver appeared to have a lower esterase activity than horse liver and the procedure as described is not applicable to beef liver.

Stage IV. Selective Heat Denaturation—The 130 ml. of solution from Stage III were brought to 63° within 1 minute in a water bath and held at that temperature for 5 minutes. The mixture was then quickly cooled in an ice bath. The precipitate was removed by centrifugation in the cold and the supernatant fluid saved. Volume 96 ml., total units 32,255, recovery for this step 75 per cent, purity 17.

Stage V. Acetone Fractionation—To 90 ml. of cold solution from Stage IV were added 30 ml. of ice-cold acetone. The resulting 25 per cent acetone mixture was allowed to stand in the cold for 12 hours, after which the precipitated proteins were removed by centrifugation and discarded. To the 106 ml. of supernatant fluid obtained were added 38.5 ml. of cold acetone, bringing the concentration of acetone to 45 per cent. After standing in the cold for 8 hours, the mixture was centrifuged, and the supernatant fluid discarded. The precipitate was thoroughly suspended in distilled water and allowed to stand for 5 hours in the cold for extraction of esterase. The mixture was then centrifuged and the clear, slightly yellow supernatant fluid was saved. Volume 38 ml., total units 18,600, recovery for this step 58 per cent, purity 41.

Stage VI. Copper Acetate Treatment and Dialysis—To the 38 ml. of solution from Stage V were added 450 mg. of solid $\text{Cu}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$ with stirring. The solution was then allowed to stand at 5° for 4 hours, after which it was centrifuged and the precipitate discarded.

The supernatant fluid (7350 units, purity 103) was dialyzed exhaustively against distilled water in the cold, and the precipitated proteins discarded after centrifugation. The solution so obtained was reduced in volume to approximately 10 ml. by a pressure filtration through cellophane.³ This solution was finally dialyzed exhaustively and the precipitated proteins discarded. Volume 11 ml., total units 5911, recovery for Stage VI 32 per cent, purity 277.

The clear and colorless solution obtained from Stage VI represents our purest esterase preparation, and contains less than 2 mg. of solids per ml. The over-all recovery was nearly 6 per cent, and represented a purification of about 270-fold over that of the acetone powder. The solution could be stored at 5° for at least 1 month without apparent loss of activity.

Properties of Enzyme

Activity toward Other Substrates—In selecting a substrate for esterase action it was found that nearly twice as much activity was shown toward

³ The apparatus used was devised by Dr. Norman Simmons of this laboratory and consists of a cellophane sausage casing contained within a strong muslin sleeve. By adequate connection with a nitrogen cylinder, 25 to 35 pounds pressure can be exerted on the solution in the cellophane bag, producing a rapid ultrafiltration.

methyl butyrate as toward ethyl butyrate. With acetylcholine bromide⁴ as a substrate, only about 2 per cent as much activity was noted as compared to methyl butyrate. We have not determined whether this slight ability to hydrolyze acetylcholine is due to the simple esterase or to slight contamination of the preparation with an acetylcholine esterase.

Optimum pH—The optimum pH of esterase action on methyl butyrate substrate was determined with borate buffers. As stated earlier, the disappearance of substrate was determined by the hydroxamate analysis of

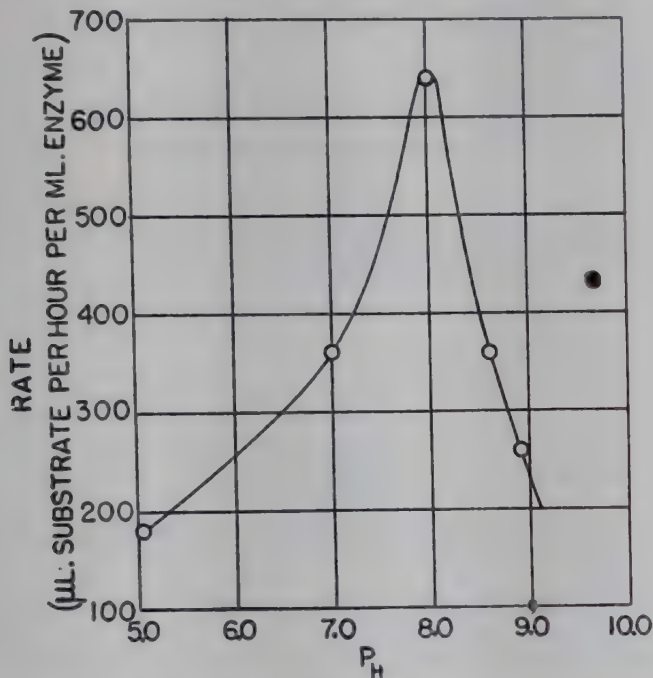


FIG. 2. Optimum pH of liver esterase with methyl butyrate substrate. 0.4 ml. of 1:200 esterase from Stage VI, 0.025 M borate buffer, and 0.1 M methyl butyrate were employed. The rates were measured by analysis of substrate (see the text) and expressed as equivalents in microliters.

residual ester. As illustrated in Fig. 2, an optimum pH of approximately 8.0 was found.

Temperature Coefficient and Energy of Activation—Rate studies were conducted over the range of 24.8–42.7° by the manometric method. The pH variations caused by this change in temperature were considered too small to affect the rate seriously. Fig. 3 illustrates the relation of rate and temperature, from which it appears that esterase is partially destroyed even at fairly low temperatures. Between 25–35° the Q_{10} was found to be 1.28. When this value was substituted in the Arrhenius equation, the energy of activation was found to be 4430 calories per mole.

⁴ We are indebted to Dr. Ralph Brauer of Louisiana State University for the sample of acetylcholine bromide.

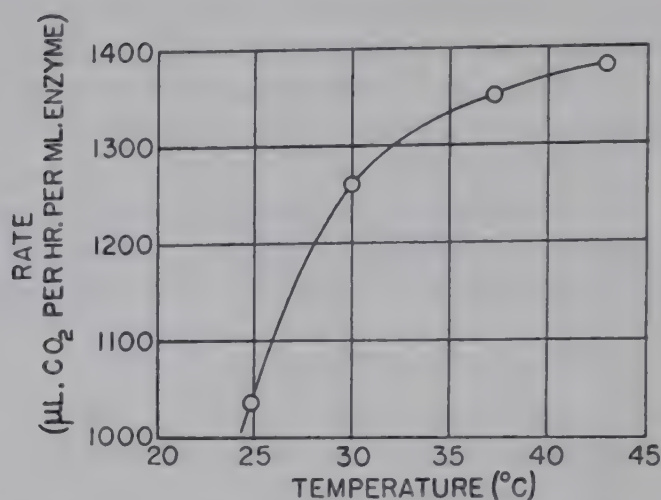


FIG. 3. Effect of temperature on rate of reaction. 0.2 ml. of 1:200 esterase preparation from Stage VI was employed. The manometric work is described under "Esterase assay."

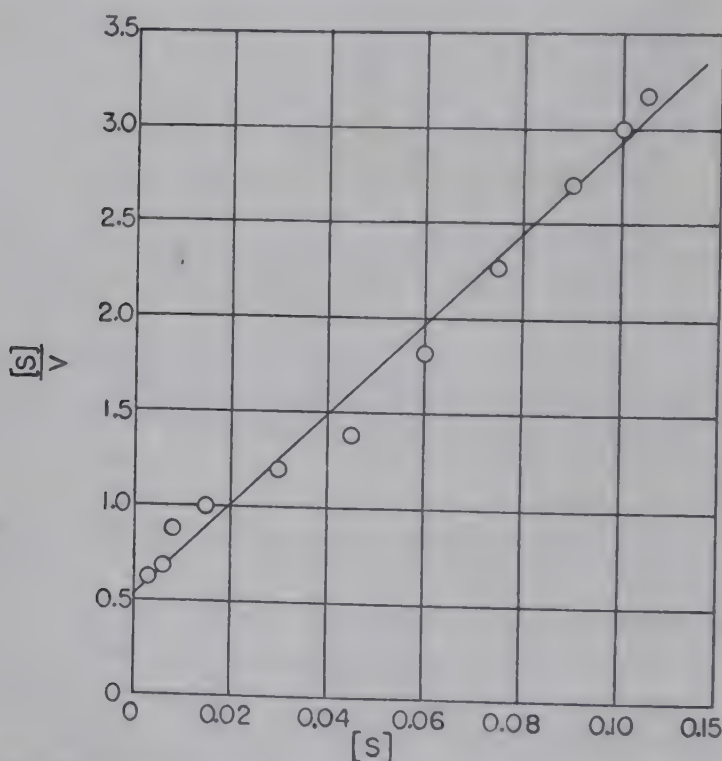


FIG. 4. Substrate concentration-velocity relations. The velocity (v) is expressed as moles of substrate hydrolyzed per liter per hour, and $[S]$ is the initial substrate concentration in moles per liter. $[S]/v = 24.115 [S] + 0.525$ as determined by the method of least squares. The manometric work is described under "Esterase assay." Esterase from Stage VI was employed.

Michaelis-Menten Constant (K_m)—The velocity of the reaction at substrate concentrations varying from 0.003 to 0.105 M was determined by the manometric method. Initial velocities (v) were expressed as moles of substrate hydrolyzed per liter of reaction mixture per hour, and substrate concentrations as moles per liter $[S]$. Fig. 4 is a plot of $[S]/v$ against $[S]$.

From the equation

$$\frac{[S]}{v} = \frac{K_m}{V_{\max.}} + \frac{[S]}{V_{\max.}}$$

where $1/V_{\max.}$ becomes the slope and $K_m/V_{\max.}$ the intercept on the ordinate $[S]/v$, the Michaelis-Menten constant K_m was calculated to be 0.022 M.

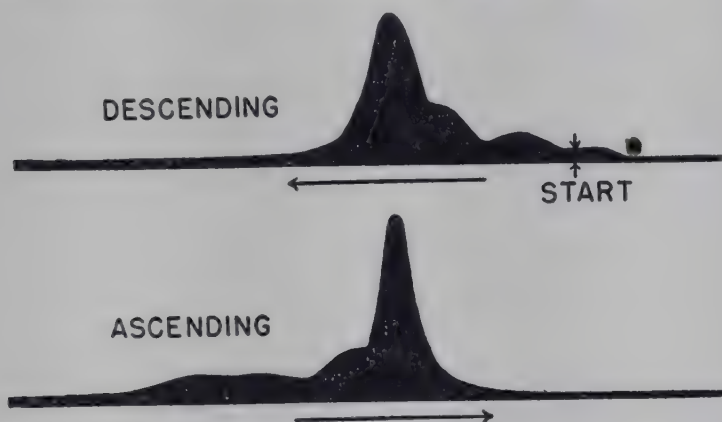


FIG. 5. Electrophoretic diagram of purified enzyme. Longsworth scanning method. Phosphate buffer, pH 7.0, ionic strength 0.1, temperature 1°, time 10,800 seconds, field strength 5.6 volts. Mobility of principal component $u = 3.4 \times 10^{-5}$ sq. cm. sec.⁻¹ volt⁻¹.

Electrophoresis—The esterase preparations were studied electrophoretically by Dr. Eric L. Alling, to whom we are greatly indebted. Fig. 5 illustrates the electrophoretic pattern of our best esterase preparation. The main component was estimated to comprise about 70 per cent of the total protein, and the component with this mobility was the one which increased with purification of the esterase.

SUMMARY

An esterase from horse liver acetone powder has been purified some 270-fold by a combination of ammonium sulfate and acetone fractionations, heat and heavy metal denaturations, and dialysis procedures. With methyl butyrate as a substrate, the optimum pH was found to be about 8.0 in borate buffer, the temperature coefficient 1.28, the energy of activation 4430 calories per mole, and the Michaelis-Menten constant (K_m) 0.022 M.

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THE UPTAKE OF GLUTAMIC ACID AND GLUTAMINE BY BRAIN AND OTHER TISSUES OF THE RAT AND MOUSE*

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Recently we reported studies of the absorption of glutamic acid and glutamine from the gut of the cat by analysis of the portal blood, and of the concentration changes of the two amino acids in the peripheral blood plasma after oral administration of glutamic acid to human subjects (3). In a further study of the fate of glutamic acid and glutamine in the animal body, the uptake of the two amino acids by brain, muscle, liver, and kidney after intravenous administration to rats and mice has now been determined. Changes in total amino acid nitrogen have been estimated previously in brain and other tissues (4, 5) after the intravenous administration of glutamic acid to rats and dogs, but this unspecific measure of amino acid concentration cannot give evidence regarding the movement of particular amino acids, especially since it has been found that a high blood level of glutamic acid causes an increased uptake of other amino acids by the tissues (3, 6).

EXPERIMENTAL

Animal Experiments—Rats (160 to 180 gm.) and mice (16 to 18 gm.) were kept without food for about 18 hours prior to the experiment. Glutamic acid (as the sodium salt), or glutamine, was injected in 7 per cent aqueous solution into the tail vein in amounts roughly corresponding to 1.2 to 1.3 mg. per gm. of body weight. Care was taken to excite the animals as little as possible during the experiment. After different time intervals, the animals were killed by decapitation and the blood was caught in weighed bottles containing 5 per cent trichloroacetic acid, which were then weighed again. In the experiments on rats the head was dropped directly into liquid nitrogen. The organs to be analyzed were dropped into liquid nitrogen within 1 minute after the death of the animal. The frozen organs were crushed (7). Muscle tissue could be obtained in

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† Fellow of the Children's Hospital Research Foundation, Washington, D. C. Under contract with the Office of Naval Research.

powdered form only with difficulty. The removal of the brain was greatly facilitated by the fact that in liquid nitrogen the rat skull including the skin cracked open longitudinally in such a way that the crack usually separated the brain into two equal halves which could be easily lifted out of the skull with the aid of a small chisel.

In the experiments on mice, the brain and other organs were removed as quickly as possible and ground in a mortar precooled with dry ice. The layer of tissue was frozen within 2 minutes after the death of the animal. The frozen rat and mouse tissues were weighed in trichloroacetic acid, as described for blood.

Preparation of Extracts and Determination—The tissue suspensions were transferred quantitatively to centrifuge tubes and centrifuged. The residues were extracted repeatedly with trichloroacetic acid, with vigorous stirring before each centrifuging. The combined extracts were transferred to volumetric flasks and diluted with water to about 90 per cent of the final volume, neutralized to pH 7, and diluted to the mark. The final concentration of trichloroacetic acid was less than 2.5 per cent. The volumes were selected so that the final concentration of glutamic acid or glutamine would be 10 to 20 γ per ml.

Glutamic acid and glutamine were determined as described previously with the modification introduced for the removal of glutathione (8, 9, 3). The fact that the ratio of glutamine to glutamic acid is much lower in the tissues than in the blood necessitated the concentration of the filtrates before hydrolysis. 2 ml. of neutralized filtrate from the "acid alumina" column (8) and 2 ml. of wash water were collected in a graduated centrifuge tube (12 ml.) and concentrated to 0.5 ml. in a boiling water bath with the aid of a gentle stream of filtered air. After the addition of 0.25 ml. of 6 N HCl, the sample was hydrolyzed for 1 hour in a boiling water bath and neutralized, and water was added to a final volume of 5 or 10 ml., according to the expected glutamic acid concentration. The glutamic acid derived from glutamine after hydrolysis was not determined directly, but was first passed through an alumina column to avoid interference by aspartic acid liberated from its amide by the hydrolysis. With tissues containing a large excess of glutamic acid over glutamine (kidney and brain), it was necessary to start with an extract which contained a multiple of the glutamic concentration suitable for the colorimetric method. This was possible, since the capacity of the adsorption column for glutamic acid exceeds by far the amounts which are within the range of the colorimetric method. Therefore, a more concentrated extract was subjected to adsorption, but only an aliquot of the acetic acid eluate was used for the ninhydrin treatment after adjustment of the aliquot to 4 ml. with 0.5 N acetic acid. By using concentrated tissue extracts and in addition concentrating the glu-

tamine-containing filtrate (see above), the glutamine values were brought within the range of the colorimetric method.

The recovery of glutamic acid and glutamine was tested after the addition of the two compounds and of glutathione to trichloroacetic acid extracts of liver and brain (Table I). Large amounts of glutathione do not interfere with the determination of the amino acid and its amide, a fact particularly important when the method is applied to liver extracts in which a concentration of glutathione at least equal to the sum of glutamic acid and glutamine is to be expected.

All determinations were carried out in duplicate with a glutamic acid standard included in every set. All values (Tables II and III) are based on

TABLE I

Recovery of Added Glutamic Acid (Acid) and Glutamine (Amide) in Extracts of Rat Liver and Brain

Values expressed as mg. per 100 gm. of tissue.

Organ	Added			Found		Calculated	
	Acid	Amide	Glutathione	Acid	Amide	Acid	Amide
Liver I				48	67		
	26	31	132	75	101	74	98
" II				47			
	36	41	180	89		83	
" III				26	55		
	47	53	235	71	106	73	108
	94	106	470	126	167	120	161
Brain				163	67		
	157	89	210	322	134	320	156
	314	178	420	490	232	477	245

determinations carried out on the tissue extracts of the pooled organs of three animals.

RESULTS AND DISCUSSION

Concentration in Organs of Fasting Animals—The glutamic acid and glutamine concentrations found in the organs of the control animals (Tables II and III) are of considerable interest, since they represent, as far as we know, the first data on the content of the two amino acids in the non-protein fraction of brain, muscle, kidney, and liver of the rat and mouse. In brain and kidney a considerable excess of the amino acid over its amide was found. The ratio in the brains of these two animals is very similar to that found with our method in cattle, rabbits, cats, and pigeons.¹ In

¹ Unpublished.

the liver, the glutamine concentration equals or exceeds that of glutamic acid, and the limited data on skeletal muscle also indicate an excess of the amide. Krebs and associates (11) recently reported on the concentration of glutamic acid and glutamine in a variety of tissues of pigeons, sheep, and cats. The determinations which have been reported are in agreement in

TABLE II

Glutamic Acid (Acid) and Glutamine (Amide) Concentration in Rat Organs after Intravenous Injection of Glutamic Acid or Glutamine

Values expressed as mg. per 100 gm.

Min.	Brain		Liver		Muscle		Kidney		Blood	
	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide
0*	152 ±16†	57 ±8.1	49 ±14	55 ±3.7	18 ±2.6	40 ±12	96 ±4.5	22 ±11	3 ±0.7	6.1 ±1.4

Glutamic acid administered

10	120	65	94	38	40	35				
10	144	52	99	31	86	58	400	29	109	5.4
15	122	58	63	69	18	42	490	38	63	7.5
15	111	34	111	65	54	55	520	43	90	7.1
20	140	56	72	45						
20	158	62	114	44			424	39	85	6.6
30	116	37					510	47	68	6.2
60	131	67					260	40	4.6	4.3

Glutamine administered

10	120	68	210	210	18	68				
15	161	78	370	92			219	99	5.1	32
15	165	85					146	239	6.7	86
20	140	84	200	73	13	53				
30	136	36	157	128			180	185	4.4	34
30	155	88					274	174	6.1	36
60	147	78					94	21	3.6	7.5

* Each average in this row represents six groups of three animals each, except for liver and muscle for which five and three groups were used, respectively.

† Standard deviation. The bold-faced values differ from the control values significantly ($P < 0.05$) (10).

showing that the brains of cattle,¹ sheep (11), cats (11),¹ dogs (12), rabbits,¹ pigeons (11),¹ rats, and mice contain about 0.01 mole of glutamic acid and about 0.004 mole of glutamine per kilo. Sheep (11) kidney contains a large excess of glutamic acid over glutamine, just as does the kidney of the rat and mouse. In the blood of rat and mouse, as in that of other mammals

studied (man, ox, and dog), a considerably higher concentration of glutamine than of glutamic acid was found.

Effect of Administration of Glutamic Acid or Glutamine on Their Concentrations in Blood—The amino acids were injected in amounts calculated to yield an initial blood concentration of 1 to 1.5 per cent, but only a small

TABLE III

Glutamic Acid (Acid) and Glutamine (Amide) Concentrations in Mouse Organs after Intravenous Injection of Glutamic Acid or Glutamine

Values expressed as mg. per 100 gm.

Min.	Brain		Liver		Kidney		Blood	
	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide
0*	169 ± 14†	72 ± 13	19	35	78	10	3.1 ± 0.6	7.2 ± 1.7
Glutamic acid administered								
15	153	80	70	32				
15	171	72					45	5.9
15	204	81			574	23	172	11
15	178	61					153	11.5
30	195	66					14	8
30	184	60					98	6.8
30	166	61					32	8.0
45	134	70					38	10.2
Glutamine administered								
15	140	116	300	90				
15	158	83			104	83	13.4	51
15	137	79					7.1	37
30	200	78			92	41	4.7	16
30	198	105					4.8	45
45	172	77					3.9	13.3

* The number of groups of three animals represented by the averages were brain (five), liver (one), kidney (one), blood (three).

† Standard deviation. The bold-faced values differ from the control values significantly ($P < 0.05$) (10).

fraction of these concentrations was found 10 or 15 minutes after the injection. The rapid disappearance of amino acids from the blood, known since the studies of Van Slyke and Meyer (13), was observed in every experiment.

In the previous study an increased concentration of glutamic acid in portal or peripheral blood was found to depress the concentration of glutamine in the plasma (3). This finding was interpreted as the result of an

increased uptake of the amide by the tissues under the influence of the elevated glutamic acid level in the blood. The phenomenon was not observed in the present experiments. The discrepancy between the earlier (3) and the present findings is not necessarily real, for glutamine was measured in the whole blood in this study and in plasma in the one reported earlier. Furthermore, the concentrations of glutamic acid were far higher in this than in the previous investigation.

The administration of glutamine led to an increase in glutamic acid concentration, presumably because of a return to the blood of the acid liberated in tissues from the amide.

Effect of Administration of Glutamic Acid and Glutamine on Their Concentrations in Brain, Liver, Muscle, and Kidney—No significant change in the concentration of either compound in the brain was found after the administration of glutamic acid, but in several experiments the concentration of the amide increased significantly after it had been given.

After glutamine injection, large increases in both glutamic acid and glutamine were found in the liver, and in some of the experiments the acid rose far more than the amide. It is reasonable to assume that the glutamic acid was liberated from the amide and that the sum of the increments of the two compounds represents the actual amount of the amide taken up and retained by the liver.

After the administration of glutamic acid the increased concentration in liver and in muscle may be explained by equilibration of the plasma concentration with that of the extracellular fluid phase. Under the conditions of the injection experiments, liver, like brain, showed an uptake of glutamine but not of the parent amino acid. It should not be overlooked that the injection of large amounts of a single amino acid creates unphysiological conditions and that different results might be obtained if glutamic acid or glutamine were administered together with and in a naturally occurring ratio to other amino acids.

After the administration of either of the two compounds, large increases in the corresponding fraction were found in the kidney. In the glutamine experiments, the increase in amide concentration was accompanied by a rise in glutamic acid.

It is probable that an inability of the brain to absorb glutamic acid is responsible for the absence of a significant increase in the concentration of either the amino acid or its amide after the administration of glutamic acid. The possibility must be recognized, however, that glutamic acid is absorbed by the brain but is metabolized at such a rapid rate that it does not increase above the normal level. Inasmuch as our analyses were carried out on whole brain without differentiation of gray and white matter, it is possible, furthermore, that a small uptake of glutamic acid by gray matter was not detected.

The high concentration of glutamic acid in brain raises the question as to the source of the amino acid. Its concentration is about twice, and together with glutamine, 3 times that of glucose. While it cannot be concluded that under different experimental conditions or in other species glutamic acid may not be found to enter the central nervous system, our experiments carried out on rats and mice suggest that the glutamic acid of the brain may have its origin partly in the amide and partly through synthesis in the brain itself. Recently Stern and associates (14) reported that glutamic acid accumulates against the concentration gradient in slices of guinea pig brain cortex suspended in a saline medium containing glucose and glutamic acid. While it cannot be excluded that species differences account for the uptake of glutamic acid by cortex slices (guinea pig) and the lack of uptake by the brain in the intact animal (rat and mouse), it is more likely that the difference in results can be ascribed to basic differences in permeability between whole organs in intact animals and tissue slices.

It may be of considerable biological significance that, as shown in our experiments, glutamine enters the liver and probably the brain with greater ease than does the parent amino acid. While glutamic acid or its keto acid takes part in many of the basic metabolic reactions (oxidative deamination, entrance of ammonia into the amino acid pool, transamination, etc.), the amide is inert in all these processes. It represents therefore a store of the metabolically highly active amino acid and keto acid from which they can be liberated easily by enzymatic action. While a change in glutamic acid or ketoglutaric acid may influence the rate of such basic metabolic mechanisms as the tricarboxylic acid cycle or transamination, a change in glutamine concentration will not affect the rate of these processes.

SUMMARY

The determination of glutamic acid and glutamine in the protein-free filtrates of brain, muscle, liver, and kidney of rats and mice with the aid of a microchemical method is described. In the whole brain of these animals, as well as in the brain of cattle, sheep, rabbits, cats, and pigeons, free glutamic acid occurs in a concentration of about 0.01 mole and glutamine of about 0.004 mole per kilo. Kidney contains a large excess of the amino acid over the amide, while in liver the concentrations of the two compounds are about equal.

The changes in concentration of glutamic acid and glutamine in blood, liver, muscle, kidney, and brain were determined at various intervals up to 1 hour after the intravenous administration of the two compounds to rats and mice.

Both amino acids disappear rapidly from the blood. After the administration of the amide, large amounts of it are taken up by the liver. The

rise in amide concentration in the liver is accompanied by the liberation of glutamic acid. After glutamic acid administration, its increase in liver and muscle may be explained by the equilibration of the blood plasma concentration with that of the extracellular fluid phase.

There was a considerable accumulation in the kidney of both compounds after the administration of either one; when an excess of glutamine was present, glutamic acid was liberated.

No significant increase in the glutamic acid or glutamine concentrations of the brain followed the injection of glutamic acid. The results of the experiments in which the amide was administered suggest that glutamine may enter the brain.

The significance of the results with respect to brain metabolism and the function of glutamine is discussed.

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EFFECT OF HIGH PRESSURES ON TRYPSIN AND CHYMOTRYPSIN*

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Investigations on the effect of pressure on enzymes may be divided into two classes: (a) the influence of comparatively low pressures (generally around 500 to 1000 bars) upon reactions catalyzed by enzymes; and (b) inactivation of enzymes by much higher pressures, such as 5000 bars and above. The work reported here falls in the second category, which includes only a few previous papers. Inactivation by pressure of yeast invertase, fungal and tree laccase (1), lipase, amylase, and trypsinogen in pancreatic juice (2), and trypsin (3) has been studied by Basset, Macheboeuf, and coworkers (4). The results show that in general high pressures caused inactivation, though only a few experiments are reported on each preparation. None of the work was done with crystalline material, which in fact was scarcely available at that time. Matthews, Dow, and Anderson (5) investigated the pressure inactivation of rennin and crystalline pepsin, particularly as affected by magnitude of pressure and duration of pressing. Coagulation or denaturation of various proteins by pressure has been studied by numerous workers. Examples are egg white by Bridgman (6) and egg albumin by Grant, Dow, and Franks (7).

The effect of pressure on enzymes and enzyme systems was inaugurated with the hope of finding another tool by means of which one protein could be separated from another and thus purified by virtue of preferential inactivation through pressure. As a start the effect of pressure on two crystalline proteinases (trypsin and chymotrypsin) has been investigated. The factors studied included pH, concentration of enzyme, magnitude of pressure, pressing time, and multiple applications of pressure.

Neither trypsin nor chymotrypsin was much affected by pressure at pH 4 and below. Above pH 4, inactivation increased with increase in pH to a maximum at about pH 7.6. The other factors were studied at pH 5 and 7.6 only. Under a given set of conditions, increase in concentration of the enzyme resulted in an increase in inactivation. For each enzyme, at a certain pH and concentration and with a constant pressing time (usually 5 minutes), there was a minimum pressure below which little or no inactivation occurred. Increase in pressure above this point resulted

* Enzyme Research Division Contribution No. 124.

in a rapid increase in inactivation up to a maximum value. Further increases in either pressure or pressing time (up to 1 hour) had little or no effect. Multiple applications of pressure resulted in a considerable increase in inactivation above that obtained at the same pressure and pressing time with one application. The effect is not due to dissolved air, for deaeration alone or followed by saturation of the enzyme solution with either oxygen or hydrogen had no effect on the inactivation of chymotrypsin. Trypsin was generally less readily inactivated by pressure than chymotrypsin. The pressure inactivation of trypsin was not reversed on standing at pH 2.0 for several hours; in this respect there is no analogy to the enzyme denatured by heat or alkali.

EXPERIMENTAL

Apparatus—The hydraulic press used was operated by a pump with a maximum pressure of 3000 pounds per sq. in.; the pressure, controlled by a relief valve, was delivered to the piston. The small piston and pressure chamber bomb were constructed according to Adams (8). The bomb was provided with a water jacket through which water from a constant temperature bath was circulated. Mineral oil was the fluid used in the pressure chamber. Pressures actually obtained within the pressure chamber were calibrated against a gage in the line by means of a piezometer (8).

Materials—The sample of crystalline trypsin used was obtained from Dr. Moses Kunitz of The Rockefeller Institute for Medical Research. A commercial preparation of crystalline chymotrypsin was used.¹

Procedure—A weighed sample of the enzyme was dissolved in buffer solution or distilled water and the pH adjusted if necessary. Two portions of the solution were transferred to plastic test-tubes, and a layer of mineral oil was added to each. One tube was introduced into the pressure chamber and mineral oil was added to the desired level. The rubber and plastic gaskets (8) were put in place, and water at 25° was circulated through the water jacket for 10 minutes. Pressure was then applied gradually over a period of about 2 minutes until the desired level was reached. The control sample was held at 25°. Both trypsin and chymotrypsin were assayed by the hemoglobin method of Anson (9). The color was measured in an Evelyn photoelectric colorimeter with Filter 660. The results were calculated as trypsin units per mg. of protein nitrogen and finally expressed as percentage retention of activity of the pressed sample as compared to the control.

Results

Influence of Rate of Application of Pressure—Three runs were made in which chymotrypsin solutions containing 0.053 mg. of protein nitrogen

¹ Purchased from the Armour Laboratories, Armour and Company, Chicago.

per ml. in 0.1 M acetate buffer, pH 5.2, were pressed for 5 minutes at 7600 bars. In the first run the pressure was applied rapidly (about 20 seconds); in the second and third runs the pressure was applied gradually over periods of 2 and 10 minutes, respectively. The pressed solutions were found to retain 48, 47, and 44 per cent, respectively, of the activity of the controls. Thus a 30-fold variation in the rate of application of pressure had no effect on the amount of inactivation. It is apparent that the heat of compression was removed from the system so rapidly that it did not influence the chymotrypsin, and that the observed effects were due to pressing and not to the heat of compression.

Permanence of Inactivation—Two experiments were carried out to ascertain whether the inactivation by pressure is permanent or not. Solutions containing trypsin and chymotrypsin (0.067 and 0.053 mg. of protein nitrogen per ml., respectively) in 0.1 M acetate buffer (pH 5.0 and 5.2) were pressed for 5 minutes at 7600 and 6200 bars, respectively. Assays were carried out as soon as possible after pressing (about 15 minutes), and also after an interval of 2 hours. The retentions of activity were 67 and 69 per cent, respectively, for trypsin, and 41 and 37 per cent for chymotrypsin. These pairs of results are in rather good agreement and indicate that the inactivation is permanent.

Influence of Dissolved Air—Several experiments were carried out on the effect of dissolved air in the inactivation of chymotrypsin by pressure. The solutions used contained 0.053 mg. of protein nitrogen per ml. of 0.1 M acetate buffer, pH 5.2. Two untreated solutions, after pressing 5 minutes at 7600 bars, retained 47 and 48 per cent, respectively, of the activity of the corresponding controls. One solution was held in a vacuum produced by a water pump until gas bubbles ceased to appear, and a layer of oil spread over the surface while the solution was still in a vacuum, and was pressed as above; 40 per cent of the activity was retained. Two other solutions were degassed in a similar manner: rapid streams of oxygen and hydrogen respectively were passed in for 3 minutes, the solutions again degassed, and oxygen or hydrogen again passed in for 3 minutes. On pressing as before, these solutions retained 46 and 48 per cent, respectively, of the activity of the controls. These experiments indicate that dissolved air was not responsible for the inactivation of the enzymes under pressure.

Influence of pH—The influence of pH on the pressure inactivation of trypsin was studied for the pH range 2.0 to 9.2, and of chymotrypsin for the range 3.1 to 7.6. A pressure of about 7600 bars was applied for 5 minutes, and the same concentration was used throughout for each enzyme. The trypsin solution contained about 20 per cent more protein nitrogen than the chymotrypsin solution. The results are presented in Fig. 1. The curves for the two enzymes are similar, although chymotrypsin was more highly inactivated than trypsin at all pH values, especially above pH 5.0.

Neither enzyme was much affected at pH 4.0 and below. Above this value the amount of inactivation by pressure increased rapidly and apparently reached a maximum at around pH 7.6. The effects of the other variables were studied only at pH values of 5.0 to 5.2 and 7.6. The activities of the control samples were practically constant for chymotrypsin; for trypsin the activities decreased slowly with increase in pH from 2.0 to 8.6. The values around pH 8.6 were about two-thirds of those around 2.0. At still higher pH values, the activities dropped to about half of that at pH 8.6.

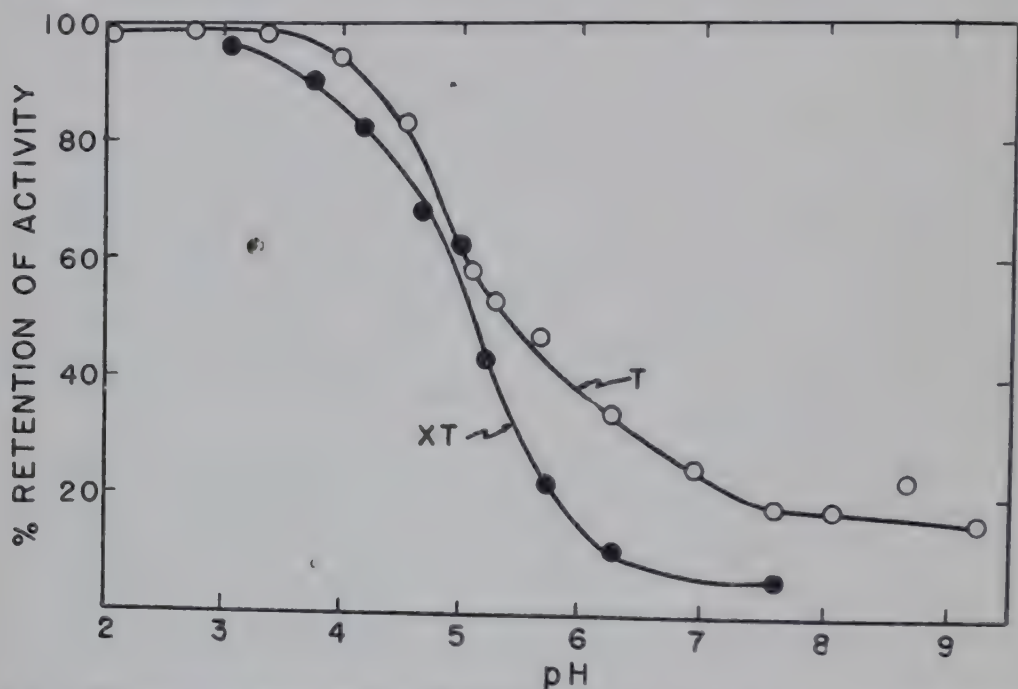


FIG. 1. The influence of pH on the inactivation by pressure of trypsin and chymotrypsin (pressure = 7600 bars; pressing time = 5 minutes; concentration of trypsin (T) = 0.067 mg. of protein nitrogen per ml., and of chymotrypsin (XT) = 0.053 mg. of protein nitrogen per ml.).

Influence of Concentration—The influence of the concentration of the enzyme upon the amount of inactivation produced by pressing at 7600 ± 100 bars for 5 minutes was tested for both enzymes at two pH values. The results are presented in Fig. 2. In every case the percentage of inactivation increased with increase in concentration. Under the same conditions, chymotrypsin was invariably inactivated to a greater extent than was trypsin. With both enzymes the amount of inactivation was much higher at pH 7.6 than at pH 5 for the same concentration.

Influence of Magnitude of Pressure—The effect of the amount of pressure at constant pressing time and concentration was investigated for each enzyme at two pH values. The concentrations, expressed as mg. of protein nitrogen per ml., were not the same for both enzymes but were of the same

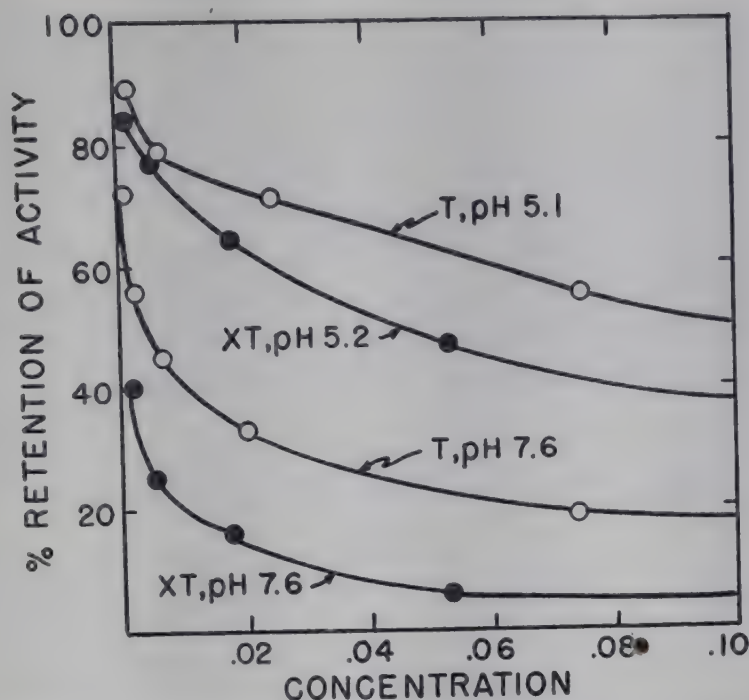


FIG. 2. The influence of concentration on the inactivation by pressure of trypsin (*T*) and chymotrypsin (*XT*) (pressure = 7600 bars; pressing time = 5 minutes; concentration expressed as mg. of protein nitrogen per ml.).

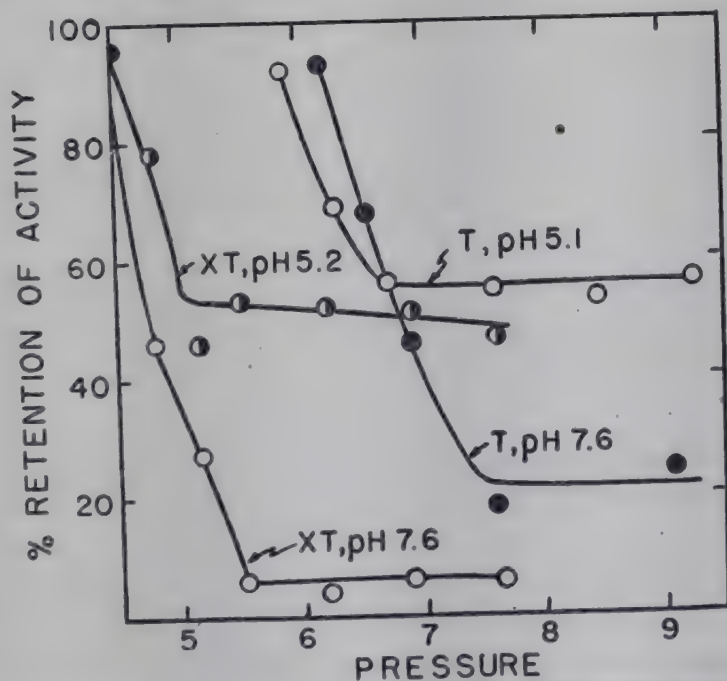


FIG. 3. The effect of pressure on trypsin and chymotrypsin (pressure in kilobars; pressing time = 5 minutes; concentration of trypsin (*T*) = 0.067 mg. of protein nitrogen per ml., and of chymotrypsin (*XT*) = 0.053 mg. of protein nitrogen per ml.).

order of magnitude. The results, as given in Fig. 3, follow a similar pattern. Above a certain point (about 4500 bars for chymotrypsin and 6000 bars for

trypsin), further increase in pressure resulted in a rapid increase in inactivation up to a second point (which varied both with the enzyme and the pH), above which considerable further increases in pressure resulted in little or no increase in the inactivation. This constant value was around 50 per cent retention of activity for both enzymes at a pH of around 5, but at pH 7.6 it was around 20 per cent for trypsin and 5 per cent for chymotrypsin. Similar experiments with chymotrypsin at a much lower concentration (0.0018 mg. of protein nitrogen per ml.) gave similar results but reached the leveling-out points at somewhat higher pressures.

TABLE I

Influence of Duration of Pressing at Constant Pressure on Trypsin and Chymotrypsin

Enzyme	Concentration	pH	Pressure	Pressing time	Per cent retention of activity
	<i>mg. protein N per ml.</i>		<i>bars</i>	<i>min.</i>	
Trypsin	0.067	5.1 <i>Ca.</i>	7500	1	52
"	0.067	5.1 "	7600	5	55
"	0.067	5.1 "	7600	60	57
"	0.067	7.6	6200	5	93
"	0.067	7.6	6200	15	63
"	0.067	7.6	6200	60	34
"	0.067	7.6	7600	1	31
"	0.067	7.6	7600	5	19
"	0.067	7.6	7600	15	20
Chymotrypsin	0.053	5.2	7600	1	59
"	0.053	5.2	7600	5	47
"	0.053	5.2	7600	15	52
"	0.053	7.6	4800	1	74
"	0.053	7.6	4800	5	46
"	0.053	7.6	4800	15	37

Influence of Duration of Pressing—A number of experiments were carried out on the effect of the duration of pressing on the inactivation of trypsin and chymotrypsin by pressure. The results, as given in Table I, show that at 7600 bars the inactivation reached a maximum in all cases within 5 minutes, and in one case within 1 minute. Neither the pressure nor the duration of pressing is at all critical here and may be varied considerably without significant effect on the amount of inactivation produced. At 6200 bars and pH 7.6, trypsin was only slightly inactivated within 5 minutes, but was about two-thirds inactivated after 1 hour, about the same as in 1 minute at 7600 bars. At 4800 bars and pH 7.6, chymotrypsin was in-completely inactivated within 5 and probably even 15 minutes. These

periments show that at pressures somewhat below 7600 bars the inactivation takes place much more slowly than at 7600 bars, and does not reach a maximum within 5 minutes.

Effect of Multiple Applications of Pressure—A number of experiments were carried out on the effect of multiple applications of pressure on trypsin and chymotrypsin. In these experiments the pressure was raised initially to 7600 bars for 5 minutes, then lowered to the minimum pressure of the press (about 400 bars) for 5 minutes, then raised again to 7600 bars for 5 minutes, etc. The data on one series of experiments at pH 5.0 for both trypsin and chymotrypsin are given in Table II. Successive applications of pressure resulted in an increase in the percentage inactivation of the enzyme, whereas single pressings for 5 and 20 minutes, respectively, yielded

TABLE II

*Effect of Multiple Applications of Pressure on Trypsin and Chymotrypsin**

Times pressure applied	Duration of pressing per application	Per cent retention of activity	
		Trypsin	Chymotrypsin
	<i>min.</i>		
1	5	67	52
2	5	47	34
3	5	37	22
4	5	31	16
1	20	64	51

* A pressure of 7600 bars was used. The enzymes were dissolved in 0.1 M acetate buffer, pH 5.0, to give a concentration in mg. of protein nitrogen per ml. of 0.067 for trypsin and 0.053 for chymotrypsin.

results in good agreement. Based on the amount of active enzyme present at the beginning of each successive application of pressure, the percentage losses of activity for the four applications were 33, 30, 21, and 16, respectively, for trypsin, and 48, 35, 35, and 27 for chymotrypsin. This decrease is to be expected since the percentage inactivation of the enzymes under a given set of conditions decreases with decrease in concentration (Fig. 2).

Several experiments were carried out with triple applications of pressure for both enzymes at pH 5.1 to 5.2 and 7.6. The results in every case showed a considerable increase in the percentage inactivation over that obtained in a single application of pressure.

Reversibility of Pressure Inactivation of Trypsin—Kunitz and Northrop (10) found that trypsin is reversibly denatured if it is heated or made strongly alkaline. The denatured trypsin is reactivated when allowed to stand at pH 2.0 at about 20° for several hours. In the present work a solution of trypsin containing 0.067 mg. of protein nitrogen per ml. (pH

5.28) was pressed for 5 minutes at 7600 bars. The pressed sample retained 53 per cent of the activity of the control. Aliquots of both solutions were made to pH 2.0 as soon as possible after the pressing, and allowed to stand at room temperature for $3\frac{1}{2}$ hours. The activity of the pressed sample was then 50 per cent of that of the control, showing that pressure denaturation is not reversible under these conditions.

Formation of Turbidity in Pressed Solutions—A turbidity after pressing was observed in a number of runs with chymotrypsin but in none with trypsin. Turbidities were observed in chymotrypsin only at pH 7.6, at pressures of at least 6200 bars, and in solutions containing at least 0.0053 mg. of protein nitrogen per ml.

DISCUSSION

Explanation of the observed effects of pressure on trypsin and chymotrypsin is not simple or obvious, especially in regard to the effect of multiple application of pressure and to the resistance to inactivation under certain conditions of a considerable part of the enzyme.

The effect of pressure varied tremendously with pH, as shown in Fig. 1. At pH 4.0 and below, pressing at 7600 bars for 5 minutes had almost no effect on either enzyme; under the same pressure conditions, both enzymes were about 50 per cent inactivated at pH 5, and much more completely so at still higher pH values. Trypsin and chymotrypsin are generally most active at around pH 8 and show little or no activity at a low pH. It is possible that the active form of the molecule is partially unfolded (11). At low pH values the molecule may assume a more globular form in which the groups responsible for the activity are less exposed. These two forms would be expected to be in equilibrium, dependent on the pH. The globular molecule should occupy a smaller volume than the partially unfolded molecule and would probably be much more resistant to pressure.

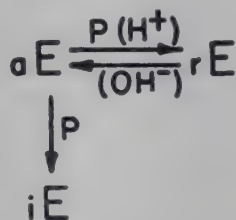
Under a given set of conditions, an increase in the concentration of either trypsin or chymotrypsin resulted in an increase in the percentage inactivation. If the solution was sufficiently dilute, no significant amount of inactivation occurred. This indicates that under the influence of pressure, an irreversibly inactivated enzyme is formed by the reaction of 2 or more molecules of enzyme, probably the partially unfolded active form.

When either trypsin or chymotrypsin was pressed at 7600 bars at pH 5.0 to 5.2, around 50 per cent inactivation was obtained within 5 minutes; pressing for periods up to 1 hour, or at pressures up to over 9000 bars, resulted in no significant increase in the inactivation. A portion of the enzyme, possibly the partially unfolded active form, is converted to the irreversibly inactive form, while the remainder is very resistant to inactivation.

tion. This part may already exist as the globular form or may be converted into this form by the high pressure.

When the solution was pressed at 7600 bars for 5 minutes, the pressure then lowered nearly to zero for 5 minutes, and again raised to 7600 bars for 5 minutes, much more inactivation occurred than during a single 5 or 60 minute pressing. When the pressure is released, the pressure-resistant globular particles would be expected to revert to the equilibrium mixture of globular and partially unfolded molecules, while the irreversibly inactivated enzyme would be unchanged. If the pressure were then applied a second time, a portion of the enzyme would be converted to the irreversibly inactivated form and the remainder to the stable globular form. Repeated application and release of pressure should eventually lead to practically complete conversion of the enzyme to the irreversibly inactivated form.

The above discussion may be summarized in the following diagram



where aE refers to the active enzyme (partially unfolded), rE to reversibly inactive enzyme (globular), and iE to irreversibly inactive enzyme. All of the results obtained with trypsin and chymotrypsin are consistent with this scheme, which is advanced as a tentative hypothesis and not as a final and complete explanation of the observed phenomena.

SUMMARY

The inactivation of crystalline trypsin and chymotrypsin by high pressures was investigated, including the effects of pH, concentration of enzyme, magnitude of pressure, pressing time, and multiple applications of pressure. Trypsin was more stable under pressure than chymotrypsin under all conditions investigated except multiple applications of pressure. The inactivation of trypsin by pressure was not reversed when it was allowed to stand at pH 2.0.

A possible explanation of the observed results is discussed.

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A RESOLUTION OF HISTIDINE, CYSTINE, AND ALANINE BY ASYMMETRIC ENZYMATIC HYDROLYSIS OF THE RACEMIC AMIDES

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A method has been described for resolving a number of racemic amino acids which depends upon the asymmetric enzymatic hydrolysis of the *N*-acylated derivatives by purified enzyme preparations from hog kidney or beef pancreas (1-5). By this means, we have obtained the optical isomers of the following amino acids conveniently, in good yield, and with a high degree of purity: alanine, α -aminobutyric acid, valine, norvaline, leucine, norleucine, isoleucine, methionine, aspartic acid, glutamic acid, lysine, serine, threonine, phenylalanine, tyrosine, and tryptophan.

We have thus resolved all of the amino acids which commonly occur in proteins, except for arginine, histidine, cystine, proline, and hydroxyproline. The method described has failed in the instances (a) of arginine and of histidine because of the difficulty of acylating both basic groups with susceptible and conveniently removed acyl radicals, and (b) of cystine and proline (and presumably of hydroxyproline) because of the complete resistance of the *N*-acylated derivatives of these amino acids to enzymatic hydrolysis by animal enzyme preparations (1, 4, 6).¹

Inasmuch as one of the purposes of the *N*-acylation procedure is to remove the dipolar character of the amino acid, it was considered that this purpose could be equally well served by removing the acid character of the carboxyl group by amide formation.² In this way, an alternative method of resolution, based upon the asymmetric enzymatic hydrolysis of the racemic amino acid amide, could be developed, which might be applicable to the cases of those amino acids for which the previous procedure based upon the *N*-acylated derivatives was not possible. Consequently, the corresponding amides of racemic histidine, *S*-benzylcysteine, and proline

¹ α -Acetyl-DL-histidine and α -acetyl-DL-arginine are asymmetrically hydrolyzed by hog kidney preparations (1). From digests of acetyl-DL-histidine with such preparations, we have been able to isolate L-histidine consistently in 70 to 80 per cent yield. It was impossible, however, to separate completely the L-histidine remaining in the mother liquor from the acetyl-D-histidine.

² Brenner, Sailer, and Kocher (7) and Brenner and Kocher (8) have employed the esters of racemic tryptophan and of methionine, respectively, as starting materials for a resolution procedure involving the use of pancreas preparations.

were prepared and subjected to the action of purified hog liver or kidney preparations. After addition of manganese ions, these preparations readily and asymmetrically hydrolyzed the amides of histidine and of *S*-benzylcysteine, and the optical isomers of these amino acids were subsequently easily isolated. Proline amide, however, was not hydrolyzed appreciably (*cf.* (9)).³

The present paper describes the preparation of the optical isomers of histidine and of cystine. A similar resolution of alanine is included for comparison.⁴

EXPERIMENTAL

The enzymatic hydrolysis of the *L* form of amino acid amides has been demonstrated by several investigators (9, 14–17). The pH of optimal susceptibility of alanine amide as well as that of several other amino acid amides toward the action of liver and kidney preparations is about 9.0 to 9.5 (17). The pH optimum for *DL*-histidine amide is about 9.5 and that for *S*-benzyl-*DL*-cysteine amide is about 9.1 (Fig. 1).

The method of resolution used in the present instance was incubation of the racemic amino acid amide at pH 8.6 to 9.2 with a purified enzyme preparation from hog liver or kidney. The liver preparation was used for the resolution of alanine, the kidney preparation for the resolution of histidine and of *S*-benzylcysteine. In either case, the *L* form of the amide was hydrolyzed to a mixture of *L*-amino acid and ammonia, whereas the *D* form of the amide remained intact (Fig. 2). The *L*-amino acid was removed from the digest, as before (1). The mother liquors which contained the *D*-amino acid amide were combined, condensed, brought to alkaline pH, and the free amide extracted into an organic solvent. Evaporation of the solvent, followed by mineral acid hydrolysis and neutralization, yielded the free *D*-amino acids. The optical rotations of the isomers obtained compared satisfactorily with those described in the literature (Table I).

³ We have observed, however, that *DL*-proline amide is readily hydrolyzed by aqueous extracts of mushrooms. Both isomers are hydrolyzed, one of them (presumably the *L* form) being cleaved much more rapidly than the other. Both isomers of *DL*-alanine amide and of *DL*-leucine amide are also hydrolyzed in similar fashion by extracts of mushrooms. The presence of an enzyme which hydrolyzes *D*-amino acid amides in this vegetable, never noted in animal tissues, will be the subject of communication elsewhere. The fact that both isomers of the amino acid amides are hydrolyzed by the mushroom, even though at different rates, makes a resolution procedure with this tissue quite difficult.

⁴ Bergmann, Fruton, and Pollok (10) have shown that α -benzoylarginine amide is readily hydrolyzed by crystalline trypsin. *L*-Arginine can be obtained directly from protein hydrolysates (11, 12) and *D*-arginine by the action of arginase on *DL*-arginine (13).

Preparation of Enzyme—The liver preparation for the resolution of alanine was made as follows. 2200 gm. of freshly frozen hog liver were allowed to thaw, ground in a Waring blender with 2 volumes of ice-cold

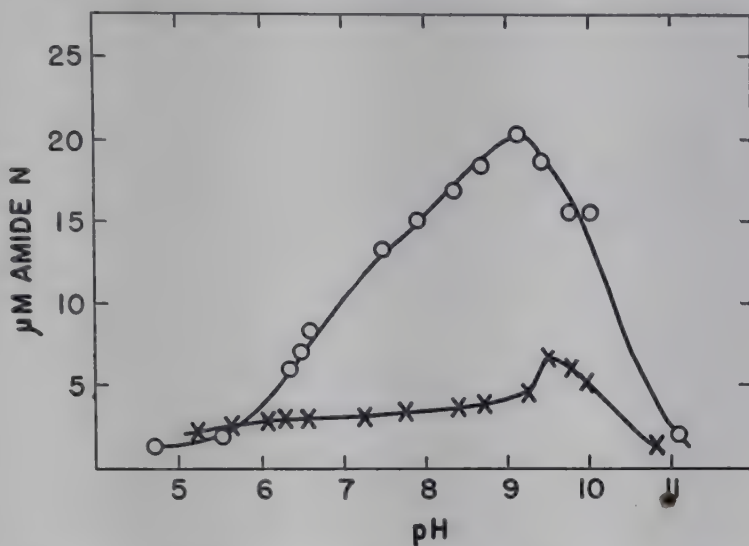


FIG. 1. pH-activity curves for histidine amide X, and for *S*-benzylcysteine amide O, with purified hog kidney preparations. Veronal-acetate buffers used below pH 8, borate buffers used above pH 8.

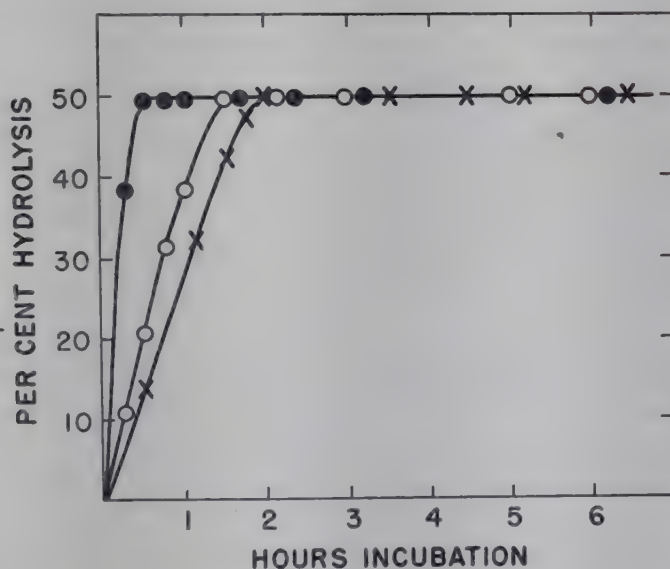


FIG. 2. Asymmetric hydrolysis of DL-alanine amide X at pH 8.6 by hog liver preparation, and of DL-histidine amide O and of *S*-benzyl-DL-cysteine amide ● at pH 9.3 by hog kidney preparation. Hydrolysis followed by ninhydrin- CO_2 measurements.

water, and strained through gauze. The extract was taken to 15 per cent alcohol, pH 6.3, and -6° , and after 12 hours was run twice through a Sharples supercentrifuge. The nearly clear supernatant was taken to 30 per cent alcohol, pH 5.3, and -15° , for 12 hours and the precipitate re-

covered in the Sharples centrifuge. This precipitate was suspended in ice-cold water, taken to pH 7.0, and stored in the refrigerator as needed. The suspension had a nitrogen content of 3.1 mg. per cc. and an activity against 0.2 M DL-alanine amide of 120 as compared to 32 in the original homogenate.⁵

TABLE I

*Specific Rotations at 25° of Optical Isomers of Racemic Amino Acids Resolved by Asymmetric Enzymatic Hydrolysis of Their Amides**

Compound	Present data					Data in literature	
	L form		D form		N calculated	L form	D form
	[α] _D	N observed	[α] _D	N observed		[α] _D	[α] _D
	degrees	per cent	degrees	per cent		degrees	degrees
Alanine.....	-14.5†	15.8	-14.4‡	15.8	15.8	+14.7 (18) +14.6 (19)	-14.5 (19)
Histidine.....	-39.7§	26.9	+39.6	27.0	27.1	-39.7 (20)	+39.8 (21)
S-Benzylcysteine..	+25.5¶	6.6	-25.0¶	6.6	6.6	+23.5** (22) +22.7 (23)	-22.5 (22)
Cystine.....	-220.0††	11.6	+221.2††	11.6	11.7	-214.4 (24)	+224.0 (22)

* 2 dm. tube employed.

† 4.82 per cent solution in 1.0 N HCl.

‡ 4.65 per cent solution in 1.0 N HCl.

§ 1.42 per cent solution in water.

|| 1.48 per cent solution in water.

¶ 1.00 per cent solution in 0.965 N NaOH.

** At a temperature of 26.5°.

†† 0.40 per cent solution in 1.0 N HCl.

The hog kidney preparation for the resolutions of histidine and S-benzylcysteine was prepared as follows. 3500 gm. of freshly frozen hog kidney were allowed to thaw, ground in a Waring blender with 7 liters of cold water, and strained through gauze. The original homogenate had an activity of 8 against 0.05 M DL-histidine amide and an activity of 22 against 0.05 M S-benzyl-DL-cysteine at pH 9.2. The extract was centrifuged at 20 minutes at 600 × g and the precipitate, P₁, discarded. Cold 60 per cent alcohol was added to the supernatant, S₁, to a final concentration of 8 per cent, the temperature being lowered simultaneously to -3°. Cold 0.5 N acetic acid was added to lower the pH to 6.3. After 12 hours the

⁵ Activities of the enzyme preparations are expressed as before (1-5) in terms of micromoles of substrate hydrolyzed per hour per mg. of N, the rate being approximated from the initial portion of the respective time curves.

precipitate, P_2 , was removed in the Sharples centrifuge and the clear supernatant, S_2 , taken to 20 per cent alcohol, pH 5.6, -9° for 12 hours. The active precipitate, P_3 , was recovered in the Sharples centrifuge, taken up in 1200 cc. of water, and adjusted to pH 7.0. This fraction, P_3 , which was employed for the resolutions, had an activity of 31 against histidine amide and 168 against *S*-benzylcysteine amide. In the presence of 0.01 M Mn^{++} , these activities were increased to 139 and 570, respectively.

DL-Alanine Amide Hydrobromide (14)—400 gm. of ethyl- α -bromopropionate were shaken to solution at 0° with 8 liters of chilled 25 per cent aqueous ammonia solution, and kept at this temperature for 4 days. The solution was evaporated to dryness and the residue crystallized twice from absolute alcohol. The *DL*-alanine amide hydrobromide was obtained in a 50 per cent yield; m.p. 175° . N calculated 16.6 per cent, found 16.6 per cent.

DL-Histidine Amide Hydrochloride—150 gm. of *DL*-histidine monohydrochloride were converted into the methyl ester dihydrochloride (*cf.* (25)) and recrystallized from methyl alcohol-ether. 120 gm. of this ester salt were dissolved in 1200 cc. of dry methanol saturated at 0° with dry ammonia. After 4 days at 20° , removal of the solvent yielded 75 gm. of *DL*-histidine amide monohydrochloride, which was recrystallized from water-acetone mixtures; m.p. 233° with decomposition. Calculated N 29.4, Cl 18.6 per cent; found N 29.4, Cl 18.3 per cent.

S-Benzyl-DL-cysteine Amide Hydrochloride—180 gm. of *S*-benzyl-*DL*-cysteine were suspended in 1800 cc. of methyl alcohol and the mixture saturated with dry HCl gas. On evaporation *in vacuo*, 135 gm. of the corresponding methyl ester hydrochloride were obtained. After crystallization from methanol-ether, the compound melted at 112° . Calculated N 5.3, Cl 13.6 per cent; found N 5.2, Cl 13.5 per cent.

120 gm. of the methyl ester hydrochloride were dissolved in 1200 cc. of methanol saturated at 0° with ammonia. After standing 4 days at 20° , the solvent was removed and the residual amide hydrochloride was crystallized from alcohol in the form of needles. The yield was 72 per cent of theory; m.p. $188-190^\circ$. Calculated N 11.4, Cl 14.4 per cent; found N 11.3, Cl 14.5 per cent.

Preparation of L-Histidine, S-Benzyl-L-cysteine, and L-Alanine—Solutions were prepared of 98 gm. of *DL*-histidine amide hydrochloride in 800 cc. of water, of 24 gm. of *S*-benzyl-*DL*-cysteine amide hydrochloride in 200 cc. of water, and of 68 gm. of *DL*-alanine amide hydrobromide in 400 cc. of water. The pH of the last mentioned solution was adjusted to 8.6 by addition of concentrated lithium hydroxide, 100 cc. of the liver preparation were added, and the pH again adjusted to 8.6. The histidine amide solution was adjusted similarly to pH 9.0, 150 cc. of the hog kidney enzyme preparation were added, manganese chloride was added to a final concen-

tration of 0.01 M, and finally enough distilled water was added to bring the total volume to 10 liters.⁶ The *S*-benzylcysteine amide solution was also adjusted to pH 9.0, 100 cc. of the hog kidney enzyme preparation were added, manganese chloride was added to a final concentration of 0.01 M, and enough distilled water to bring the total volume to 2 liters. In all cases, enough enzyme preparation was added to bring about the theoretical maximal hydrolysis in 2 to 4 hours.

The digests were allowed to stand at 37° for several hours. Aliquots were removed from time to time in order to follow the hydrolysis by manometric-CO₂ analyses. To be completely sure of the completion of hydrolysis, more enzyme was added to each digest, and the digestion allowed to proceed for several hours beyond the point at which maximal hydrolysis (50 per cent of the racemate) had occurred.

At the end of the digestion period, the digests were each brought to pH 5 by addition of glacial acetic acid, shaken with and filtered through norit, and evaporated *in vacuo* to a small volume. During this evaporation the *S*-benzyl-L-cysteine crystallized; the crystals were filtered off and dried. The yield of this compound was 10.3 gm., or 95 per cent of theory. The condensate from the histidine amide digest, about 200 cc., was brought to pH 7.0 by addition of lithium hydroxide, and hot absolute alcohol was added to 80 per cent. The crystals of L-histidine were filtered off, washed with alcohol, and dried. The yield was 32 gm. or 84 per cent of theory. The condensate from the alanine amide digest, about 100 cc., was treated directly with 800 cc. of hot absolute alcohol, and the resulting crystals of L-alanine filtered off, washed with alcohol, and dried. The yield was 14 gm., or 78 per cent of theory.

The crude *S*-benzyl-L-cysteine was dissolved in a little warm dilute HCl, treated with norit, and filtered. The filtrate was chilled and treated with dilute ammonia to pH 6.0. The hexagonal prisms of the pure compound were filtered, washed thoroughly with water, and dried; yield 6.0 gm. The crude L-histidine was recrystallized from a little hot water with the aid of norit, and yielded 18 gm. of pure, dried product. The L-alanine was recrystallized twice from water-alcohol mixtures, each time with the aid of a little norit, and finally 10.0 gm. of pure dried product were obtained. The optical and analytical data on these purified preparations are given in Table I.

Preparation of D-Histidine, S-Benzyl-D-cysteine, and D-Alanine—The mother liquors and washings from the respective preparations of the L-amino

⁶ We have noted that the complete enzymatic hydrolysis of histidine amide occurs rapidly only in dilute solutions. It is likely that this is due to inhibition of the enzyme by products of the reaction. Further studies of this phenomenon are in progress.

acids were each combined, evaporated *in vacuo* to a small volume, and chilled to 5°. Ice-cold 5 N sodium hydroxide was added dropwise to each concentrate until the pH was about 11. 4 liters of acetone were then added, the mixture shaken thoroughly, and the acetone layer decanted and filtered. The residual aqueous layer of each concentrate was extracted three times in similar fashion with acetone. The respective acetone extracts were combined, evaporated nearly to dryness, and again extracted with acetone. This procedure was repeated until no turbidity occurred, when fresh acetone was added to the previous condensate. The acetone extracts were then evaporated *in vacuo* nearly to dryness, taken up in about 5 times the amount of 3 N hydrobromic acid, and refluxed for 1 hour. The solution was treated with norit, filtered, and evaporated *in vacuo* to dryness.

The hydrolysate containing *S*-benzyl-D-cysteine was dissolved in a little water, filtered, and treated with dilute ammonia to pH 6.0. The resulting crystals were filtered, washed thoroughly with water, and dried; yield 6.0 gm., or 56 per cent of theory. The D-histidine hydrolysate was dissolved in a little water, the solution brought to pH 7.0 with dilute ammonia, and hot absolute alcohol added to 80 per cent. After filtering and drying the resulting crystals, the yield was 8.2 gm. Recrystallization from hot water yielded 6.2 gm. of pure D-histidine, or 17 per cent of theory. A second preparation of D-histidine gave no better yield than this, and we are at a loss to account for the unusually low yield of this amino acid. The D-alanine hydrolysate was dissolved in a little water, brought to pH 6.0 with dilute ammonia, and treated with hot absolute alcohol to 80 per cent. Repetition of this procedure yielded 8.0 gm. of pure, dried product, or 44 per cent of theory. The optical and analytical data on these purified preparations are given in Table I.

Conversion of Isomeric S-Benzylcysteines to Corresponding Cystines—The *S*-benzyl-L-cysteine and *S*-benzyl-D-cysteine were converted to the corresponding isomeric cystines by the sodium-liquid ammonia procedure of Wood and du Vigneaud (22). The optical and analytical data for these isomers are given in Table I.

The authors wish to acknowledge the skilful assistance of Mrs. Rembert B. Kingsley.

SUMMARY

The optical enantiomorphs of histidine, *S*-benzylcysteine, and alanine have been prepared by asymmetric enzymatic hydrolysis of the respective amides. L-Histidine with $[\alpha]_D = -39.7^\circ$ and D-histidine with $[\alpha]_D = +39.6^\circ$, *S*-benzyl-L-cysteine with $[\alpha]_D = +25.5^\circ$ and *S*-benzyl-D-cysteine with $[\alpha]_D = -25.0^\circ$, and L-alanine with $[\alpha]_D = +14.5^\circ$ and D-alanine with

$[\alpha]_D = -14.4^\circ$ were obtained. The isomeric *S*-benzylcysteines were converted, respectively, to L-cystine with $[\alpha]_D = -220.0^\circ$ and D-cystine with $[\alpha]_D = +221.2^\circ$.

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INHIBITION OF THE GROWTH OF THE RAT BY L-PENICILLAMINE AND ITS PREVENTION BY AMINOETHANOL AND RELATED COMPOUNDS*

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Penicillamine (β,β -dimethylcysteine) is a degradation product of all the known naturally occurring penicillins (1). It was first reported in 1943 (2). Since that time most investigations have dealt with its preparation, chemical properties, or relationship to penicillin, and little information is available regarding its biological behavior. The purpose of the present paper is to report the results of an investigation of penicillamine as a possible metabolic antagonist. Some of these results have been reported in a preliminary note (3).

Since penicillamine bears a structural resemblance to a number of naturally occurring amino acids, particularly cysteine, methionine, valine, and threonine, it occurred to us that penicillamine might act as a metabolic antagonist toward one of these amino acids. We found that addition of L-penicillamine to the diet did inhibit the growth of young rats on a 20 per cent casein diet and eventually led to death of the animals. However, cysteine, valine, and threonine failed to overcome this inhibition. The effect of methionine upon the response to penicillamine was equivocal and appeared to be affected by the presence of choline in the basal diet. When choline was removed from the diet, additional methionine did not counteract the effect of penicillamine. However, when choline was added to the diet in sufficient amounts without the additional methionine, the effects of penicillamine were completely overcome.

To determine whether or not the quaternary ammonium structure was essential for this reversal of growth inhibition induced by penicillamine, dimethylaminoethanol and monomethylaminoethanol were investigated and were found to be as effective as choline. It then occurred to us that perhaps the methyl groups were not essential for the antipenicillamine activities of these compounds. Aminoethanol itself was tested and found to be even more effective than choline in overcoming the growth-inhibiting effects of L-penicillamine.

The administration of L-penicillamine to rats was frequently followed by

* The authors wish to thank the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this work.

the appearance of running fits and clonic and tonic convulsions, symptoms which could be precipitated by exposing the animals to loud or high pitched sounds. Aminoethanol and its *N*-methyl derivatives prevented the appearance of these symptoms.

It was of particular interest to compare the effect of *D*-penicillamine with that of the *L* isomer, since it is the *D*-penicillamine which is obtained when naturally occurring penicillins are degraded (1). It was found that *D*-penicillamine did not inhibit growth or produce the nervous symptoms associated with the action of *L*-penicillamine. This marked difference in the effect of these two enantiomorphs offers a striking example of stereochemical specificity.

TABLE I
Choline-Free Basal Diet

Vitamin-free casein.....	20 gm.
Sucrose.....	55 "
Salt mixture*.....	4 "
Vitamin mixture†.....	1 "
<i>D,L</i> -Methionine.....	0.15 gm.
Corn oil‡.....	1.0 ml.
Vitamin A and D concentrate§.....	0.5 drop
Hydrogenated vegetable oil.....	19 gm.

* Osborne and Mendel (8).

† The vitamin mixture contained thiamine hydrochloride, riboflavin, nicotinic acid, pyridoxine hydrochloride, and *p*-aminobenzoic acid, 1 mg. each, calcium *d*-pantothenate 5 mg., inositol 10 mg., biotin 0.01 mg., folic acid 0.1 mg., and sucrose to make 1 gm.

‡ Corn oil containing 4.0 mg. of α -tocopherol acetate and 0.1 mg. of 2-methyl-1, 4-naphthoquinone per ml.

§ From pharmacy of New York Hospital, 5 drops = 120 mg.; 60,000 i.u. of vitamin A per gm., 10,000 i.u. of vitamin D per gm.

A comparison was also made between *L*-penicillamine and the corresponding disulfide. When *L*-penicillamine disulfide was fed to young rats, it did not inhibit their growth. Furthermore, *S*-methylpenicillamine showed no growth-inhibiting properties.

EXPERIMENTAL

Composition of Diets—The composition of the basal diet is given in Table I. A choline-free basal ration was used. To this basal ration the various compounds to be tested were added at the expense of an equal weight of sucrose. When either *L*-penicillamine hydrochloride hydrate or choline chloride was used, 1 equivalent of sodium bicarbonate was also added to the diet.

Growth Experiments—Young male albino rats of the Rockland strain were used for all experiments. The animals were fed the basal ration *ad libitum* for 1 week before being placed on the experimental diets. The average weight gain was about 25 gm. during this period.

Effect of L-Penicillamine upon Growth—When a level of 0.35 per cent L-penicillamine hydrochloride hydrate was added to the diet, immediate weight loss and decrease in food consumption occurred. In the course of studying a variety of compounds for their ability to counteract the action of penicillamine, forty-two animals have been fed the penicillamine diet as controls for periods up to 10 weeks. Thirty-seven of them died within the 10 week period; twenty-two of them died after they had received the penicillamine for 4 weeks or less. Forty of the rats lost weight during the 1st week following the addition of penicillamine to the diet. The weight losses ranged from 5 to 38 gm., with an average loss of 20.6 gm. In subsequent weeks the animals which survived generally lost weight more slowly or showed erratic weight changes. The average daily food consumption on the basal ration was 8.8 gm. per rat; it fell to 3.3 gm. during the week following the addition of penicillamine to the diet.

Fits or convulsions were observed in twenty-three of the rats. These symptoms did not usually appear until the animals had been receiving L-penicillamine for at least a week. No gross pathological symptoms were consistently observed at autopsy.

Effect of Choline and Related Compounds on Growth of Penicillamine-Treated Animals—After exploratory experiments had been conducted with aminoethanol, monomethylaminoethanol, dimethylaminoethanol, and choline, an experiment was carried out in which the four compounds were tested simultaneously. The animals were fed the basal ration for the 1st week, and for the 2nd week the diet containing 0.35 per cent L-penicillamine hydrochloride hydrate. After this period, an appropriate level of the compound to be tested was added to the diet containing penicillamine. The growth curves of these animals, shown in Fig. 1, illustrate the prompt growth response to the choline-ethanolamine series of compounds under these conditions.

Another type of experiment was performed in which the preventive substance and penicillamine were added simultaneously to the diet. Aminoethanol and its *N*-methyl derivatives were again found to be effective in overcoming the growth-inhibitory action of L-penicillamine. Of the thirty-six animals tested, six served as controls receiving penicillamine alone. Although three of the control animals died before the end of the 10th week, none of the rats receiving the preventive compounds did so. When the *N*-methyl compounds and penicillamine were added to the diet, temporary cessations of growth lasting for a few days were observed in every instance

before the resumption of growth comparable to that of animals on the basal diet in the absence of penicillamine. However, no inhibition of growth

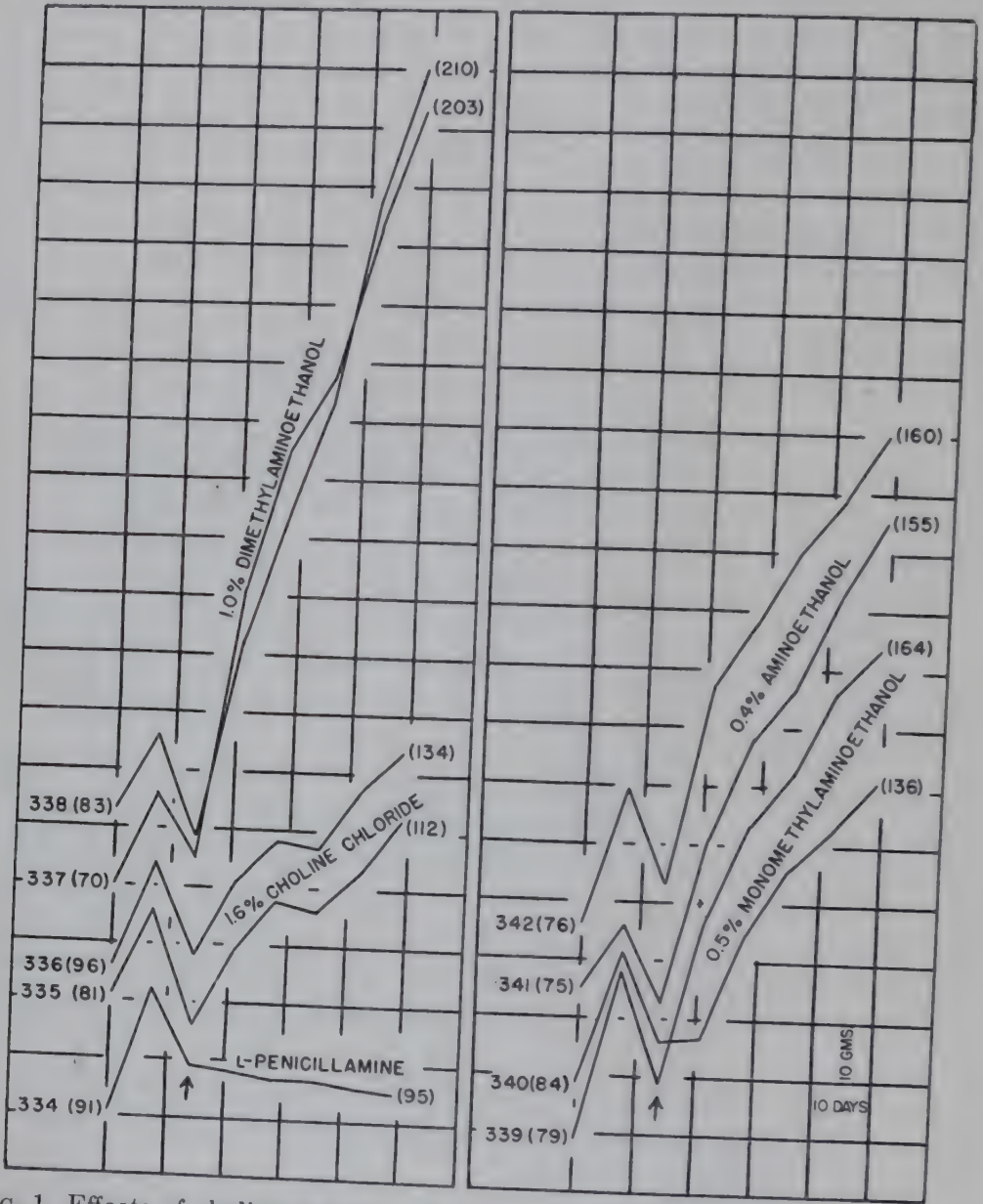


FIG. 1. Effects of choline and related compounds upon the growth inhibition due to L-penicillamine. The animals were male litter mates; 0.35 per cent L-penicillamine hydrochloride hydrate was added to the basal diet (Table I) on the 7th day; the compounds to be tested were added on the 14th day (arrow) at the levels indicated. The numbers in parentheses indicate initial and final weights.

occurred when aminoethanol and penicillamine were added simultaneously to the diet.

Effect of D-Penicillamine upon Growth—Both curative and preventive experiments demonstrated clearly that D-penicillamine did not possess the

growth-inhibitory power of the L form. Fig. 2 shows the growth curves from a typical experiment. Further investigation showed that D-penicillamine produced no observable toxic effects, even when large doses (660 mg. per kilo) were injected intraperitoneally into rats. Fits, convulsions, and

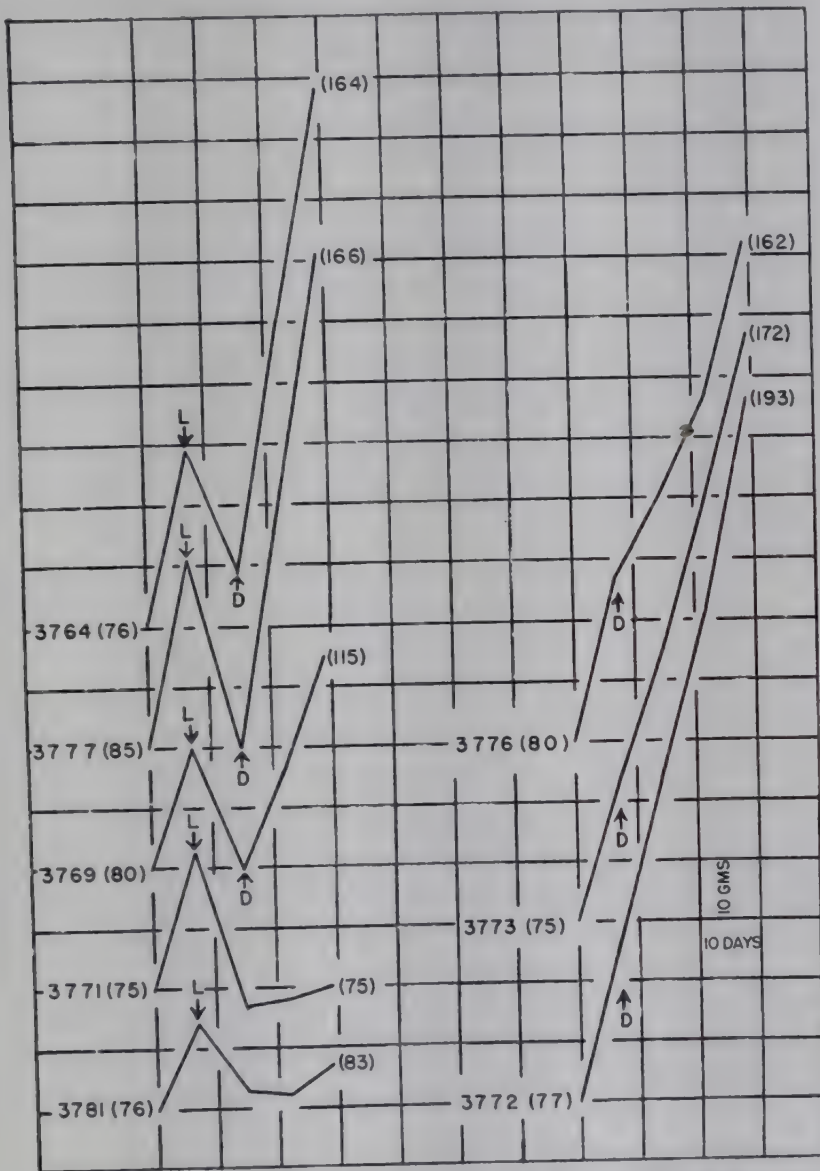


FIG. 2. Effect of D- and L-penicillamine upon growth. L indicates the addition of 0.35 per cent L-penicillamine hydrochloride hydrate to the basal diet (Table I); D indicates the addition of 0.35 per cent of the D isomer to the basal diet. The numbers in parentheses indicate initial and final weights.

death almost invariably followed the injection of half this dose of the L isomer.

Effect of L-Penicillamine Disulfide upon Growth—When L-penicillamine disulfide was added to the basal diet at a level of 0.27 per cent, no breaks

were observed in the growth curves of the rats to whose diets it was added. When the level of the disulfide was doubled, there was still no effect on growth.

Effect of S-Methyl-DL-penicillamine upon Growth—When *S*-methyl-DL-penicillamine was added at a level of 0.5 per cent to the diets of rats, no significant growth inhibition occurred over a period of 2 weeks. Neither *S*-methylpenicillamine nor penicillamine disulfide produced the nervous symptoms associated with the administration of *L*-penicillamine.

Sources of Compounds—The aminoethanol and dimethylaminoethanol used in these experiments were Eastman products and were fractionally distilled before use. The choline chloride was a gift from the American Cyanamid Company. To prepare monomethylaminoethanol, the method of Schotte, Priewe, and Roescheisen (4) was modified by dissolving the phosgene in toluene and carrying out the first two steps of the synthesis in this solvent. This eliminated the sealed tube reaction used in the original method. The *L*-penicillamine disulfide was made by a procedure previously described (5). The methods used for making *D*- and *L*-penicillamine and *S*-methylpenicillamine are given in detail.

D- and L-Penicillamine Hydrochloride Hydrate—These compounds were prepared by a modification of the procedure described previously (6) for the preparation of *D*-penicillamine hydrochloride. 50 gm. of *S*-benzyl-*N*-formyl-*L*-penicillamine¹ ((5) p. 462) and 9.6 gm. of sodium were added alternately in small portions, with stirring, to liquid ammonia (800 ml.). At the end of the reduction, the blue color owing to the excess sodium was discharged upon the addition of ammonium chloride (27 gm.). The ammonia was then evaporated by gently warming the mixture, the last traces being removed at water pump pressure. The residue was dissolved in 150 ml. of water and the resulting solution was extracted with three 50 ml. portions of U. S. P. ether. The aqueous layer was then cooled to 10° and made acid to Congo red with 6 *N* hydrochloric acid. After the mixture had been allowed to stand for 1 hour in an ice-salt bath, the precipitate was removed by filtration, washed with a little cold water, and pressed dry on the filter.

This *N*-formyl-*L*-penicillamine was dissolved in 180 ml. of 1 *N* hydrochloric acid by heating, and then heated under a reflux for 1 hour. The resulting yellow solution was decolorized completely with Darco, filtered, and evaporated under reduced pressure. The crystalline residue was dissolved in 50 ml. of commercial absolute ethanol and filtered. The filter was washed with a little ethanol. The combined filtrate and washings were

¹ The authors are indebted to Parke, Davis and Company for placing at their disposal a supply of *S*-benzyl-DL-penicillamine, which served partially as a source of *S*-benzyl-*N*-formylpenicillamine used in this investigation.

then added in small portions, with scratching and stirring, to 1800 ml. of ether containing several drops of concentrated hydrochloric acid. After this mixture had been allowed to stand overnight at 8°, a crystalline product was removed by filtration, washed with a little cold ether, and dried *in vacuo* over sulfuric acid. A yield of 30.2 gm. of L-penicillamine hydrochloride hydrate (79 per cent of the theoretical amount) was obtained; $[\alpha]_D^{22} = +46^\circ$ (1 per cent in 1 N sodium hydroxide).

D-Penicillamine hydrochloride hydrate, prepared in an identical manner, had a rotation of $[\alpha]_D^{21} = -45^\circ$ (1 per cent in 1 N sodium hydroxide).

S-Methyl-DL-penicillamine—This compound was made by the following modification of the methionine synthesis of Patterson and du Vigneaud (7). *S-Benzyl-DL-penicillamine* (20 gm.) was reduced in 300 ml. of liquid ammonia with 4.5 gm. of sodium. The blue color owing to the excess sodium was discharged with 0.5 ml. of methyl iodide, and an additional 5.5 ml. of methyl iodide were added with stirring. After the mixture had been stirred for an additional 5 minutes, the ammonia was allowed to evaporate, the last traces being removed by gentle warming at water pump pressure. The residue was dissolved in 50 ml. of water and extracted with a little ether. A 15 per cent aqueous solution of hydriodic acid was then added until the aqueous layer was barely alkaline to litmus. After the resulting mixture had stood overnight at 5° the crystalline product was removed in a sintered glass funnel, washed with a little cold water, and dried *in vacuo* over phosphoric anhydride. An additional small amount of product was obtained by concentration of the mother liquors. The total yield was 12.8 gm. (94 per cent of the theoretical amount) of a product possessing a micro melting point of 250°, with some darkening at 220°. After two recrystallizations from water, and drying *in vacuo* at 80° over phosphoric anhydride, the product was analyzed.

$C_6H_{13}O_2NS$.	Calculated.	C 44.15, H 8.02, S 19.64
163.2	Found.	" 44.14, " 8.18, " 19.84, 19.55

SUMMARY

L-Penicillamine has been fed to growing albino rats and has been found to cause loss of weight and death in these animals. The toxicity of this compound is also displayed by the appearance of peculiar nervous symptoms in animals to which it is administered. These responses to the inclusion of L-penicillamine in the diet are prevented by the addition of amino-ethanol or any of its *N*-methyl derivatives. The influence of structure upon the toxicity described above was investigated in experiments with D-penicillamine, penicillamine disulfide, and *S*-methylpenicillamine. None of these three compounds was found to inhibit the growth of the rat.

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OBSERVATIONS CONCERNING THE PRODUCTION AND EXCRETION OF CHOLESTEROL IN MAMMALS

I. PLASMA CHOLESTEROL AFTER BILE DUCT LIGATION AND FREE CHOLESTEROL INJECTION*

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Investigators (1-6) are agreed that in man, the dog, and the rat biliary obstruction is followed by a rise in the total cholesterol concentration of the blood. Opinion is not uniform concerning the changes in blood content of esterified cholesterol after biliary obstruction. Thus, Epstein and Greenspan (3, 4) observed a rise in cholesterol ester concentration of the blood in the majority of their patients who suffered from obstructive jaundice, whereas Man *et al.* (6) observed a reduction in the concentration of esterified cholesterol in a group of similar patients. Chanutin and Ludewig (5) found, at most, slight change in the esterified cholesterol content of the plasma of rats subjected to ligation of the bile duct.

The mechanism responsible for the rise in the free cholesterol content of blood plasma following biliary obstruction is not clear. It was felt that further information concerning this mechanism, and concerning the formation of esterified cholesterol, might be gained if animals with ligated bile ducts were subjected to an acute additional cholesterol load. The latter was accomplished by the intravenous injection of a specially prepared suspension of free cholesterol stabilized with sodium laurate (7).

Methods

Male and female rats (albino and Long-Evans strains), weighing between 140 and 200 gm., were used. One series of 50 rats was operated upon under ether anesthesia and the bile duct ligated. Blood samples (1 ml.) were obtained 6, 24, and 72 hours after ligation and the plasma was analyzed for free and total cholesterol. A control series of twelve rats was anesthetized, the abdominal cavity opened, and the bile duct manipulated in a laparotomy. Blood samples were obtained and the plasma analyzed exactly as in the larger group.

A series of twenty-six rats was operated upon as above, the bile duct ligated, and immediately after ligation the rats were given, by intravenous injection, a suspension of 2.6 per cent free cholesterol prepared as reported

* Aided by a grant from the American Foundation for High Blood Pressure.

previously (7). Each rat received 20 mg. of cholesterol per 100 gm. of body weight. Blood samples were obtained and the plasma analyzed as described below. For control purposes, a series of twenty-four rats was anesthetized, and a laparotomy performed without actual ligation of the bile duct. These rats received the same amount of cholesterol as the preceding rats, and blood samples were obtained and treated as described for the experimental animals.

All rats subjected to ligation of the bile duct were examined at autopsy, and data from those found with ruptured biliary ducts were discarded. None of the rats received food for the duration of the experiments.

The total cholesterol concentration of blood plasma was analyzed according to the method of Saifer and Kammerer (8) with the following modification. Irregularities in color development were decreased when the reagent grade acetic anhydride used was redistilled and when the final solution was cooled in the refrigerator prior to the addition of 0.25 ml. of H_2SO_4 and incubation at 37.5° for color development.

The method of analysis for free cholesterol was the same as that used previously (7). As details of the method were not set forth in the previous paper, they are described here. A 1:1 mixture of alcohol-acetone is added in a rapid stream directly to a measured volume of clear plasma in a lipped tube suitable for centrifuging. 1.5 ml. of alcohol-acetone are used for each 0.1 ml. of plasma. The resultant finely divided precipitate is mixed by rotation, centrifuged, and the supernatant fluid decanted through a paper filter. The precipitate and paper are washed with alcohol-acetone. The extract and washings are combined, the volume noted, and one-half this volume of 0.56 per cent digitonin in 47 per cent ethanol is added. The precipitate is allowed to form at room temperature for 16 hours, centrifuged, the supernatant fluid discarded, and the cholesterol digitonide washed twice with 3 ml. of ether. The digitonide is dried at 60° , and 4.5 ml. of acetic anhydride-dioxane mixture (8) are added. The digitonide is dissolved in the mixture by heating the tube to approximately 140° for 30 minutes, and the mixture cooled and analyzed according to the procedure of Saifer and Kammerer (8), with modifications as described above for the total cholesterol determination. Comparison is made with a standard solution of free cholesterol and with a blank.

Results

Change in Free and Esterified Cholesterol Following Ligation of Bile Duct— The average free cholesterol concentration of plasma was higher 6 hours after ligation of the biliary duct than it was in rats subjected to laparotomy alone, and it continued to rise at 24 and 72 hours (Table I). No appreciable change occurred in the control rats.

In contrast to the behavior of free cholesterol, there was little or no rise

in cholesterol ester concentration of the plasma of these same rats after ligation of the bile duct.

Change in Free and Esterified Cholesterol Following Ligation of Bile Duct and Intravenous Introduction of Free Cholesterol—The free cholesterol of the plasma reached the highest concentrations observed in this study in rats which received an injection of cholesterol immediately after ligation of the duct (Table I). At each of the intervals studied, the average concentration exceeded that found in rats with ligated ducts, which had not received cholesterol, by about the same amount; namely, 65 to 71 mg. per 100 ml. In control rats which had received the same quantity of cholesterol after

TABLE I

Rise in Plasma Cholesterol Content after (a) Ligation of Bile Duct and (b) Ligation of Bile Duct Plus Intravenous Injection of Free Cholesterol

	No. of rats	Time after operation	Total cholesterol per 100 ml. plasma		Free cholesterol per 100 ml. plasma		Esterified cholesterol per 100 ml. plasma	
			Average	Standard error of mean	Average	Standard error of mean	Average	Standard error of mean
Ligation of bile duct	50	hrs. 6	mg. 69	mg. 2.2	mg. 28	mg. 1.6	mg. 41	mg. 1.6
	48	24	125	4.0	81	5.7	44	3.0
	33	72	278	16.9	215	14.9	63	5.0
Simple laparotomy	9	6	51	6.1	14	2.5	37	3.3
	9	24	47	5.7	13	3.0	34	2.6
	12	72	45	4.3	10	1.6	35	3.2
Ligation of bile duct plus cholesterol injection	23	6	148	9.1	97	8.1	51	3.5
	24	24	197	12.8	152	11.4	47	2.5
	26	72	365	25.8	280	21.5	85	5.9
Simple laparotomy plus cholesterol injection	24	6	86	4.3	45	4.2	41	2.5
	23	24	77	5.7	40	3.1	37	3.4
	21	72	54	3.2	17	1.7	37	2.4

simple laparotomy there was a much smaller increase at 6 and 24 hours, with a return, almost to the normal level, at 72 hours.

The plasma cholesterol ester concentration of rats with ligated ducts, which had received an injection of cholesterol, was not significantly changed either at 6 or 24 hours after ligation, but at 72 hours it was significantly higher than the concentration found in the plasma of such rats which had not received cholesterol after ligation (Table I).

DISCUSSION

A rapid and large increase in the free cholesterol component of blood plasma of rats after ligation of the biliary duct, without a quantitatively

comparable rise in esterified cholesterol, was observed in our study. These results are essentially similar to those of Chanutin and Ludewig (5). They suggest the possibility that the plasma level of free cholesterol in the rat may be maintained, at least in part, by means of a hepato-biliary mechanism, while the cholesterol ester content of blood is relatively independent of such a mechanism.

If the data in Table I are plotted, the slope of the line representing free cholesterol concentration in the plasma of rats with ligated ducts is found to be constant and practically the same as that of the corresponding line for rats injected with free cholesterol after ligation. This shows clearly that the rate of entrance of free cholesterol into the plasma, possibly the rate of production, is constant and independent of its plasma concentration. The unvarying amount (65 to 71 mg. per 100 ml.) by which the free cholesterol in plasma of rats, after both ligation and injection, exceeds that following ligation, even after a period of 72 hours, seems quite remarkable for a substance which has on occasion been thought to be an active metabolite. It is possible that the free cholesterol injected cannot be metabolized. This possibility seems somewhat unlikely in view of the greater rise of ester cholesterol (at 72 hours) in animals following ligation and injection compared with ligation alone, the rapid disappearance of injected cholesterol from the blood stream of the intact rat (7), and the probable partial conversion of injected cholesterol to the esterified form in intact animals as previously reported (7). The persistence in the blood stream of the excess 65 to 71 mg. per 100 ml. of free cholesterol after injection suggests that the regulation of free cholesterol in the blood plasma of the rat does not occur at the site of its formation, release, or possible absorption, but rather at the site of its elimination or destruction. This site may be in the hepato-biliary system.

SUMMARY

The concentration of free and esterified cholesterol in the blood plasma of rats subjected (a) to ligation of the bile duct, or (b) to ligation of the bile duct followed by a single intravenous injection of a free cholesterol suspension, was observed at intervals 6, 24, and 72 hours following ligation.

It was found that simple ligation of the bile duct alone effected a rapid and great increase in the free cholesterol of plasma, with a much slower and lesser rise in esterified cholesterol. Animals ligated and injected with free cholesterol showed a greater rise in free cholesterol. The injected excess cholesterol did not affect the rate of increase of free cholesterol attributable to the biliary obstruction, but remained as a fixed increment superimposed upon the sustained rise which results from simple obstruction.

The possibility is suggested that regulation of the free cholesterol level

in the plasma of the rat is dependent upon its destruction or excretion by the hepato-biliary system.

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FIXATION OF N_2^{15} BY EXCISED NODULES OF LEGUMINOUS PLANTS*

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As symbiotic nitrogen fixation requires both a host plant and an effective strain of the *Rhizobium*, it would seem that the seat of fixation is the specific organ of symbiosis, the nodule. Surprisingly, mere excision of the nodule apparently results in the loss of this highly important function. The earlier reports were contradictory (1), but this might be explained as arising from the general inadequacy of the technique available. Application of the isotopic method by Wilson and his associates (2-5) provided a reliable analytical technique. In the comprehensive trials made with this method, positive findings have been obtained in about 10 to 15 per cent of the tests. Their occurrence has been so erratic that it was suggested that they might have arisen from an uncontrolled factor, such as a nitrogen-fixing contaminant (4). Experiments to test this possibility are reported in this paper.

Methods

Two methods for studying the proposal of Machata *et al.* (4) appeared promising. The first is to grow plants under usual experimental conditions and then to make careful bacteriological examinations in all manipulations. If nitrogen-fixing contaminants are frequently present, then the second method should be attempted: test of nodules from plants grown free of all microorganisms except the *Rhizobia*. The first method was followed in this study, since all previous investigations had used the non-sterile technique but had failed to mention exercising any bacteriological examination during the nitrogen fixation tests.

Soy bean and cow-pea plants, inoculated with an effective strain of *Rhizobium*, were grown in a nitrogen-poor sand supplemented with mineral salts. The pots were kept in a greenhouse or in an outside cold-frame; no particular efforts were made to maintain sterile conditions other than those usually employed in this type of work. When the plants were actively fixing nitrogen, the nodules were picked into ice water, and their surface was sterilized. A 7 per cent solution of calcium hypochlorite for 5 min-

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utes was tried but was unsatisfactory, as the chlorine penetrated the nodule and killed the bacteria. Treatment with a 1:10,000 dilution of Roccal¹ for 5 minutes, however, reduced materially the load of bacteria on the outside without apparent harm to those inside. The nodules were then washed thoroughly in sterile distilled water and placed directly in the flasks used for the experiments with the isotope. These flasks contained only enough mineral solution to cover the bottom, allowing direct contact between the nodules and the atmosphere. In some trials, the outer walls of the nodules were crushed in a sterile mortar to increase the oxygen supply of the bacteria. Other additions, e.g. organic supplements, were made aseptically.

Leaves from the plants after removing the nodules were disintegrated with a Waring blender in about 6 times their weight of water. The solid material was removed in the centrifuge and the supernatant extract filtered through a Seitz filter. Nodular "paste" was prepared by a thorough crushing of the nodules in sterile distilled water, expressing the juice through cheese-cloth and removing the water-soluble fraction ("pigment") with a Beams' centrifuge. The paste was washed in sterile water and again recovered in the Beams' centrifuge. Samples of nodules, both whole and crushed, leaf extract, and paste were always tested for anaerobic and aerobic organisms capable of using free nitrogen. The anaerobic organisms were detected with Winogradsky's nitrogen-free medium incubated at 30° under an atmosphere of N₂. Anaerobic growth, gas production, and characteristic morphology constituted a positive test. *Azotobacter* was tested for by aerobic growth in Burk's nitrogen-free medium and by its typical morphology. Plates of yeast water-sucrose-mineral agar established the presence of *Rhizobium* and nodule contaminants requiring combined nitrogen. The details of the isotopic method have been described (2).

Results

Table I summarizes the analyses made with the mass spectrometer, and Table II supplies certain details of the experiments. Among the variables tested was the addition of α -ketoglutaric acid as well as an extract of leaves as possible sources of undefined photosynthetic intermediates required for fixation. Oxalacetic acid had been tried unsuccessfully (4). Because of the claim of Krascheninnikov (1) that raising the partial pressure of oxygen induces fixation of free nitrogen by excised nodules, the pO_2 was raised to 0.8 atmosphere in some trials.

Significantly, spore-forming anaerobes of the genus *Clostridium* were consistently detected. The nodules were suspended in sterile Allison's

¹ A high molecular alkyl dimethylbenzylammonium chloride, Winthrop-Stearns, Inc., New York.

TABLE I
 N_2^{15} Fixation by Excised Nodules

Type of sample	Experiment No.	pO_2	Supplements*	
			None	α -Ketoglutarate
Whole nodules	1	atmosphere 0.0	0.044	0.013
	2	0.2	0.050	0.040
	3	0.2	0.029	0.028
	4	0.8	0.006	
			0.013	
	5	0.8	0.009	
Crushed nodules			-0.002	
	6	0.8	0.053	0.046
	1	0.0	0.043	
	2	0.2		0.023
	3	0.2		0.018
	4	0.8	0.014	0.000†
			-0.005	0.008†
	5	0.8	0.009	0.003†
			0.006	-0.003†
	6	0.8	0.056	
	7	0.2	0.037	
Paste	8	0.2	0.001	
	9	0.2	0.042	
	7	0.2	0.028‡	
Washed paste	9	0.2	0.132	
	10	0.2	0.069	
	7	0.2	0.067	
	8	0.2	0.028	
Ground paste			1.19	
	9	0.2	0.094	
			0.137	
	10	0.2	0.071	
	11	0.2	0.137	
	11	0.2	0.012	
			0.008	

* Figures in atom per cent N^{15} excess; 0.050 atom per cent excess indicates positive fixation. The pN_2 was 0.2 atmosphere.

† Supplemented with leaf extract in place of α -ketoglutarate.

‡ The use of hypochlorite may have caused this negative result, since the same preparation when washed did fix N^{15} . In all other experiments (except Experiment 7) Roccal was used in place of hypochlorite.

solution; during the experiment this turned cherry-red through leaching of the hemoglobin pigment and developed cloudiness and at times an odor of putrefaction. Microscopic examination of the fluid revealed spore-

forming Gram-positive rods, becoming Gram-negative with age; enrichment cultures prepared by passage through Winogradsky's nitrogen-free medium were capable of fixing molecular nitrogen (as much as 2 per cent excess N^{15}). Although Beijerinck stated in 1922 that he could not grow leguminous plants aseptically and reported *Clostridium pasteurianum* as a common contaminant of the nodule, this observation appears to have been neglected in evaluating the results from fixation experiments with excised nodules.

Although detection of nitrogen-fixing anaerobes was not difficult, their presence seldom led to positive findings when the entire nodule was tested

TABLE II
Experimental Details of Runs with Isotopic Nitrogen

Experiment No.	Atom per cent N^{15} excess		Source of nodules	Mannitol†
	Initial gas	Control*		
1	5.90		Soy bean	—
2	6.95	0.011	" "	—
3	6.95	0.011	Cow pea	—
4	9.50	—0.014	Soy bean	—
5	6.90	—0.017	Cow pea	—
6	6.59		Soy bean	—
7	8.85	—0.016	Cow pea and soy bean	+
8	9.40		Cow pea	+
9	10.8	—	Soy bean	+
10	3.23		" "	+
11	10.6		" "	+

* An air-grown sample containing a normal percentage of N^{15} is used for a control of the mass spectrometer.

† 1 per cent mannitol added to basal medium, Allison's salts, including 3 mg. of Fe and 0.1 mg. of Mo per ml. Since there is sufficient carbohydrate in the nodules, addition is not considered to be essential.

(Experiments 1 to 9). Only three definitely positive results were obtained, all between 0.05 and 0.06 per cent excess N^{15} , not very striking examples of fixation. If, however, the water-insoluble material of the nodule was separated from the soluble portion, the resulting paste, which included most of the bacteria but little soluble nitrogen, definitely fixed N_2^{15} in nearly all the tests (Experiments 7 to 11). Such positive cultures contained numerous *Clostridia* together with the prevalent *Rhizobia*. No fixation was obtained with the soluble fraction alone, a combination of the soluble fraction and paste, or with the paste after mechanical grinding for 1 hour. Supplementary causes of the inability of the contaminated whole nodules to give consistent positive results probably include removal of

most of the contaminants by washing with the surface-sterilizing agent and the effect of free oxygen. Elimination of oxygen in one trial, however, did not induce fixation.

DISCUSSION

Over 150 samples have been tested in three independent surveys of nitrogen fixation by excised nodules (3, 4), and though all of the conditions claimed to induce fixation have been included, the results, in general, have been negative, with occasional erratic positive findings. Although these positive findings may actually represent fixation by the excised nodule, an alternative explanation is the presence of a nitrogen-fixing contaminant, together with the chance use of nodules low in soluble nitrogen.² In support of this view we frequently detected the nitrogen-fixing anaerobe, *Clostridium*, in nodules grown in open containers under usual greenhouse conditions. Even though *Clostridium* is present, the results with whole nodules are seldom positive, probably because of the presence of excess soluble nitrogen. Removal of the water-soluble fraction allows fixation to occur. From these results it appears that reports, including our own, of positive fixation by excised nodules cannot now be regarded as decisive because of the suspicion that the nodules may have harbored nitrogen-fixing anaerobes. Until more convincing data are presented, then, it seems doubtful that excised nodules under the conditions so far tested are able to fix N_2 .

SUMMARY

The nodules from soy bean and cow-pea plants grown in nitrogen-poor sand under the usual non-sterile conditions in the greenhouse or outside cold-frame frequently contain species of the nitrogen-fixing anaerobe, *Clostridium*. Surface sterilization of the nodule, the technique commonly used to insure purity of culture in nitrogen-fixing tests with nodules, does not always accomplish this.

Although *Clostridium* may be present, excised nodules do not ordinarily fix enough nitrogen to be detected even by the sensitive isotopic method

² It is noteworthy that in our own experiments nodulated roots usually fixed nitrogen. This result may have a physiological basis or may be only a reflection of a higher probability for a heavy initial load of contaminants. In one such experiment both nodules and nodulated roots from cow-peas grown in aseptic cultures fixed appreciable quantities of N_2^{15} (5). Even this observation is equivocal, however, since purity of culture rests on the assumption that our technique for growing the plants free of contamination was successful. In the absence of an adequate bacteriological control during the actual nitrogen fixation tests, such an assumption is at least questionable.

with N_2^{15} . Presence of oxygen, numbers of contaminants, and especially supply of soluble combined nitrogen apparently control the process.

The presence of nitrogen-fixing contaminants of the nodule, such as *Clostridium*, could explain the erratic and seemingly contradictory results reported by different investigators. Although some of these may have actually secured fixation by excised nodules, the lack of adequate bacteriological control seriously impairs the significance of the experiments. Proof of fixation and, even more important, definition of the necessary experimental factors that will insure its occurrence are not as yet realized experimentally.

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THE MONOIODOTYROSINE CONTENT OF THE THYROID GLAND*

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In 1929, Harington and Randall (2) attempted to account quantitatively for all of the iodine in the thyroid gland. Employing a many stage fractionation procedure, they succeeded in isolating crystalline thyroxine and crystalline diiodotyrosine from desiccated thyroid. Although the amount of iodine recovered in these two compounds was much less than that contained in the starting material, the losses encountered at each step of the fractionation procedure were not beyond those to be expected from the procedure used. Harington and Randall, therefore, concluded that all of the organic iodine in the thyroid gland could be accounted for by two compounds: thyroxine and diiodotyrosine.

This view was generally accepted until Fink, Dent, and Fink (3), applying the method of filter paper partition chromatography to thyroid hydrolysates from rats injected with radioactive iodine, found evidence for the existence of other iodine compounds in the thyroid. Fink and Fink (4) later reported that one of the spots observed in their autographs corresponded exactly with the position occupied by added moniodotyrosine and they concluded that moniodotyrosine is a normal component of thyroid tissue. The possibility that the moniodotyrosine was formed during the hydrolysis, however, was not ruled out with certainty.

The present communication deals with the question of whether moniodotyrosine is actually a normal component of the thyroid gland. A quantitative procedure for the determination of moniodotyrosine in thyroid hydrolysates by means of filter paper partition chromatography is also presented.

EXPERIMENTAL

Most of the experiments reported here were carried out on rat thyroids, but some experiments with bird thyroids are also reported. Male rats of the Curtis-Dunning or the Long-Evans strains, weighing 200 to 250 gm., were used. Those of the former strain were raised on a diet containing 2.5 γ of iodine per gm., those of the latter, on a diet containing 1 γ per gm.

* A preliminary report on this topic has appeared (1).

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The rats were injected with doses of radioiodine varying from 50 to 250 μ c. Dosage depended upon the interval elapsing between the time of injection and the time of sacrifice. Thyroids from four rats were pooled for treatment as described below.

The birds used were white Leghorns weighing 1000 to 1500 gm. They were injected with approximately 150 μ c. of carrier-free I^{131} and sacrificed 24 hours later.

Treatment of Tissue—The thyroid tissue obtained from four rats was weighed and the iodine separated into organic and inorganic fractions. This was accomplished by grinding the tissue in a glass homogenizer with 1 cc. of 10 per cent trichloroacetic acid (5). The homogenate was centrifuged in a 15 cc., graduated conical tube, and the precipitate containing the organic iodine was washed first with 2 cc. of 2.5 per cent trichloroacetic

TABLE I

Butanol Extractability of Iodine in Thyroid Hydrolysates Adjusted to pH 3.0

Animal	Hydrolytic agent	Iodine found in		Per cent of hydrolysate iodine extracted with butanol
		Butanol extract	Residue	
Rat	8% $Ba(OH)_2 \cdot 8H_2O$	64.6*	1.1	98
"	8% "	78.1*	3.4	96
"	2 N NaOH	41.8†	0.95	98
"	2 " "	50.2†	1.1	98
Chicken	2 " "	35.8†	0.25	99

* Four extractions with butanol, the first with 3 cc., the others with 2 cc.

† Four extractions with butanol, each with 3 cc.

acid and then with 5 cc. of distilled water. The trichloroacetic acid supernatants and washings were combined; these contained the inorganic fraction. •

Hydrolysis of Organic Fraction and Extraction of Iodine—The precipitate containing the organic iodine fraction was hydrolyzed on a steam bath for 16 hours with either 1 cc. of 2 N NaOH (6) or 1 cc. of 8 per cent $Ba(OH)_2 \cdot 8H_2O$ (7). The results in both cases were quantitatively quite similar (Table IV). The hydrolysis was carried out in the same centrifuge tube in which the separation described above was made. During the hydrolysis, a cold finger condenser was inserted into the centrifuge tube to prevent volume loss.

The pH of the cooled hydrolysate was adjusted to 3.0 by the addition of 6 N HCl in the case of the barium hydroxide hydrolysis, or 6 N H_2SO_4 in the case of sodium hydroxide hydrolysis. The acidified hydrolysate

was then repeatedly extracted in the centrifuge tube with butyl alcohol. The mixture was centrifuged after each extraction and the clear butanol layer was drawn off by suction with a fine tipped, capillary tube. It was found that four extractions, each with 2 or 3 cc. of butanol, succeeded in removing approximately 98 per cent of the iodine in the hydrolysate (Table I). The total volume of the combined butanol extracts amounted to 12 to 15 cc. By this procedure, the labeled iodine compounds were freed from a large part of the non-iodine-containing materials which would have interfered with the subsequent fractionation.

Quantitative Filter Paper Chromatography

In the early stages of this investigation, two-dimensional filter paper chromatography was employed, but it soon became apparent that such a procedure was not suitable for quantitative work. It was not possible to account for more than 60 per cent of the added iodine with such a method. The single dimensional procedure described below was finally developed. It was suitable for quantitative work, and provided good separation of the iodine-containing components of the thyroid.

Technique of Filter Paper Fractionation—Strips of Whatman No. 1 filter paper, 10 × 36 cm., were cut from the large commercial sheets, and a faint pencil line was drawn 2.5 cm. from one end. This guide line, 5 cm. long, extended between points 2.5 cm. from each edge of the paper. The butanol extract containing the thyroid iodine was then applied, in a thin band along this line, with a 25 μ l. pipette. Successive 25 μ l. applications of the butanol solution were made on the line, each being allowed to dry before the next was added. In this manner, it was possible to accumulate 250 μ l. or more of the butanol solution on the paper. In the case of the pooled rat thyroids, this amount of solution contained approximately 1 γ of iodine.

The filter paper strips were then suspended in large, cylindrical Pyrex jars (8½ inches in diameter and 18 inches in height) containing 150 cc. of solvent. The filter paper strips were hung from plate glass which also served as covers for the jars. The strips were suspended, by paper clips, from a stainless steel rack attached to the plate glass. The bottom of the filter paper strips extended a few mm. below the surface of the solvent; which traveled up the paper by capillary action.

The solvent usually consisted of a collidine-water mixture prepared by adding 44 cc. of water to 125 cc. of redistilled collidine (Koppers Company, b.p. 172° corrected).¹ A small beaker containing concentrated NH₄OH was placed in the bottom of the glass jar to provide an atmosphere of am-

¹ Commercial collidine, even after distillation at 172°, varies in its composition, as shown by the quantity of water necessary to form a saturated solution.

monia. The jars containing the filter papers were placed in a large, thermostatically controlled box, and the solvent was allowed to travel for 16 hours at 25–26°. At the end of that time, the papers were removed and dried in a hood for about 1 hour.

In some experiments, a mixture of butanol-acetic acid-water (200:30:75) was used as solvent. In such cases, no ammonia was used.

Location of Known Iodine Compounds on Filter Paper by Chemical Means—At the beginning of the work, it became necessary to determine the position taken on the filter paper, under the conditions described above, by pure samples of iodine compounds which might be found in the thyroid gland. The iodine compounds tested were moniodotyrosine, diiodotyrosine, thyroxine, and 3,5-diiodothyronine. Each compound was added in excess to a separate hydrolysate which was then extracted with butanol. The amount added provided about 15 to 30 γ of the compound in the total amount of solution delivered to the filter paper. The resulting chromatogram was dried and then sprayed, first with 2.5 per cent Na_2CO_3 solution and next with an aqueous solution of sulfanilic acid diazonium chloride. All the iodine compounds tested here gave sharply defined, colored bands under these conditions.

In the amount of solution delivered to the filter paper, the quantities of these various iodine compounds contributed by the thyroid tissue itself were insufficient for their location on the chromatogram by the color test. Therefore, we resorted to the use of radioautographs of the chromatogram in order to locate the various iodine fractions of thyroid hydrolysates to which no pure compounds were added.

Location of Radioactive Iodine Compounds of Thyroids by Radioautograph of Filter Paper Chromatogram—The dried chromatogram was placed in an x-ray film holder with a sheet of no screen x-ray film. The film was developed after a suitable interval, and the positions of the radioactive bands were compared with those of the known compounds as determined by color tests. Definite, reproducible bands (Fig. 1) were observed which corresponded exactly with the positions of added diiodotyrosine, moniodotyrosine, and thyroxine as located colorimetrically. This correspondence was observed not only when the collidine solvent was used, but also when the butanol-acetic acid-water mixture was used. A fourth band, corresponding to inorganic iodide, was also present in all experiments. Other weak bands were observed in some experiments and their significance remains to be established.

Once the major bands of the autographs had been identified, it was no longer necessary to add the known compounds to the thyroid hydrolysate. Instead, radioautographs were made of each chromatogram. The positions of the various iodine components on the filter paper chromatogram

were determined by superimposing the filter paper on the autograph in exactly the same position applied during the exposure. The filter paper was then marked off in sections corresponding to the bands on the auto-

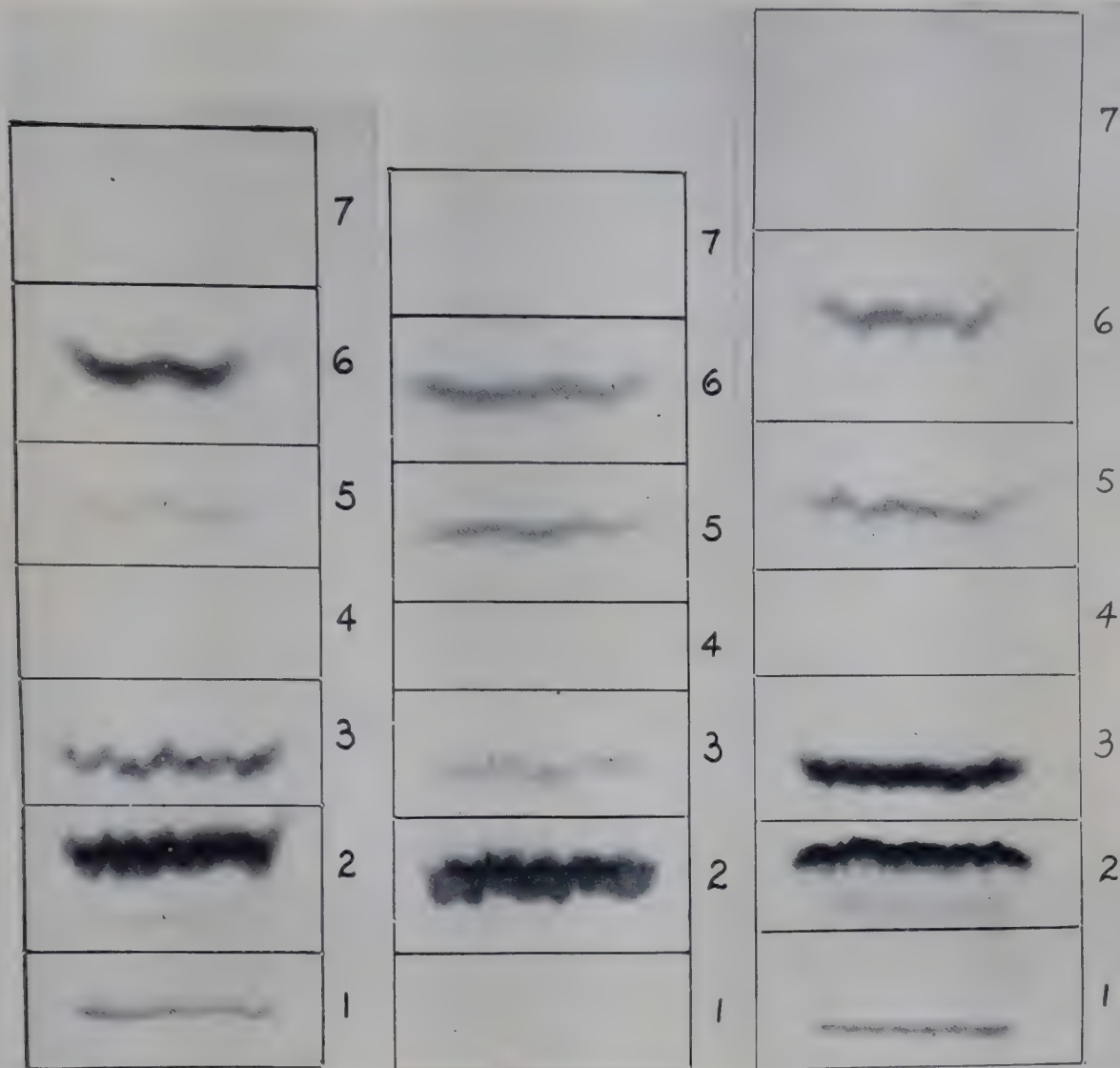


FIG. 1. Left, radioautogram of ascending chromatogram prepared from thyroids of rats injected for 10 minutes with I^{131} ; exposure time 19 hours. Center, from thyroids of rats injected for 24 hours with I^{131} ; exposure time 3 hours. Right, from thyroid of chicken injected for 24 hours with I^{131} ; exposure time 5 hours.

graph (Fig. 1), and each section was analyzed for iodine. The iodine method employed in this laboratory (8) was admirably suited to this purpose, since it makes use of a chromic acid digestion procedure which allows the filter paper sections to be digested without further treatment. The iodine is reduced with phosphorous acid, distilled, and trapped in

dilute NaOH. Both chemical and radioactive iodine were determined on the final distillate.

Test of Efficiency of Filter Paper Partition Technique—Ten large rats which had been maintained on a low iodine intake were injected with I^{131} , and their thyroids were removed 4 hours later. Five samples were prepared from these glands, each sample consisting of the thyroids of two rats (approximately 70 mg. of tissue). The thyroid samples were homogenized with trichloroacetic acid and the washed precipitates were hydrolyzed with 1 cc. of 2 N NaOH on the steam bath for 16 hours. Known quantities of pure iodine compounds were added accurately to the cooled hydrolysates in 50 μ l. of alkaline solution. Recrystallized DL-thyroxine (Squibb) was added to Tube 1, recrystallized diiodo-L-tyrosine to Tube 2, and crystalline monoiodo-DL-tyrosine (prepared by Sir Charles Harington) to Tube 3. Tubes 4 and 5 were untreated and served as controls. The hydrolysates were adjusted to a pH of 3.0 and were then repeatedly extracted with butanol as described above. Analysis of the residues remaining after BuOH extraction revealed that less than 0.1 per cent of the added iodine failed to be extracted.

250 μ l. of each butanol extract were delivered to separate filter paper strips, and chromatograms were prepared with collidine-water solvent in an atmosphere of NH_3 as previously described. The papers were dried and exposed to x-ray film for several hours. The resulting autographs served as a guide for cutting up the chromatograms into sections corresponding to the various iodine components of the gland. These sections of filter paper were then analyzed for their I^{127} content. The recoveries of added iodine in each section are shown in Table II. These recoveries are corrected for the iodine contributed by the thyroid tissue as determined in the control.

It is evident that the added iodine is almost quantitatively recovered in the appropriate filter paper section. The deviation from 100 per cent recovery may be due to several factors: (a) breakdown of iodine compounds during the partition on filter paper, (b) incomplete purity of the added standard compounds, and (c) inherent difficulties in the partition method itself.

Destruction by Hydrolysis; Recovery of Known Crystalline Iodine Compounds Added before Hydrolysis—In order to obtain some estimate of the degree of breakdown of thyroxine, diiodotyrosine, and monoiodotyrosine during the hydrolysis, known quantities of these compounds were added to thyroid tissue immediately before hydrolysis with 2 N NaOH. The hydrolysis, butanol extraction, chromatography, and iodine analyses were carried out exactly as described above. The results are shown in Table III. It is clear, by comparison with Table II, that considerable breakdown

TABLE II

Recovery of Pure Thyroxine, Diiodotyrosine, and Monoiodotyrosine Added to Hydrolysate of Rat Thyroid

Section of chromatogram (see Fig. 1)	Component represented	Iodine initially present (control)*	Recovery of added iodine†					
			6.10 γ thyroxine iodine added		5.16 γ diiodotyrosine iodine added		3.57 γ monoiodotyrosine iodine added	
		γ	γ	per cent	γ	per cent	γ	per cent
1		0.01	0.08	1.3	0.01	0.2	0.02	0.5
2	Diiodotyrosine	0.03	0.05	0.8	4.99	99	0.04	1.0
3	Monoiodotyrosine	0.015	0.03	0.5	0.08	1.6	3.50	98
4		0.01	0.06	1.0	0.01	0.2	0	0
5	Thyroxine	0.02	5.88	96	0.04	0.8	0.05	1.4
6	Inorganic	0.02	0.21	3.4	0.12	2.4	0.12	3.4
7		0.01	0.26	4.3	0.10	2.0	0.06	1.7
Total...			6.57	107	5.35	106	3.79	106

* The rats used in this experiment were raised on a low iodine intake. Their thyroids contained 9.5 mg. per cent of iodine.

† All values have been corrected for the iodine initially present (control).

TABLE III

Recovery of Pure Thyroxine, Diiodotyrosine, and Monoiodotyrosine Added before Hydrolysis of Rat Thyroid

Section of chromatogram (see Fig. 1)	Component represented	Iodine initially present (control)*	Recovery of added iodine†					
			12.1 γ thyroxine iodine added		11.0 γ diiodotyrosine iodine added		5.90 γ monoiodotyrosine iodine added	
		γ	γ	per cent	γ	per cent	γ	per cent
1		0.17	0.23	1.9	0.17	1.5	0.05	0.8
2	Diiodotyrosine	0.34	0.59	4.9	9.46	86	0.09	1.5
3	Monoiodotyrosine	0.17	0.39	3.2	0.21	1.9	5.4	92
4		0.058	0.34	2.8	0.04	0.4	0.03	0.5
5	Thyroxine	0.19	8.31	69	0.10	0.9	0.05	0.8
6	Inorganic	0.21	1.93	16	0.37	3.4	0.19	3.2
7		0.11	0.36	2.9	0.39	3.5	0.16	2.7
Total...			12.2	100.7	10.7	97.6	5.97	101.5

* The rats used in this experiment were raised on a high iodine intake. Their thyroids contained 100 mg. per cent of iodine.

† See the foot-note to Table II.

of added thyroxine occurs during hydrolysis with 2 N NaOH, amounting to 20 to 25 per cent of the added thyroxine. The breakdown of added mono- and diiodotyrosine is considerably less (less than 10 per cent).

Similar experiments, in which 8 per cent $\text{Ba}(\text{OH})_2$ was used for hydrolysis, revealed somewhat less breakdown of *added* thyroxine (about 18 per cent), but slightly more breakdown of added monoiodotyrosine.

Results

Typical autographs obtained in several runs with the thyroids of rats and chickens injected with I^{131} are shown in Fig. 1.² The solvent employed in preparing the chromatograms was collidine-water in an atmosphere of NH_3 . The four major iodine components of the thyroid stand out very clearly. These are, with increasing distance from the origin, diiodotyrosine, monoiodotyrosine, thyroxine, and inorganic iodide. Faint bands are also noticeable near the origin and near the advancing solvent front. The latter may represent breakdown products arising during hydrolysis.

The filter paper chromatograms were divided into seven sections as shown in Fig. 1, and each section was analyzed for its I^{127} and I^{131} content. The I^{127} contents and the specific activities of the various fractions as determined with this procedure are shown in Table IV. The I^{127} recoveries varied from 112 to 116 per cent; the high recoveries are probably due to a small filter paper blank. The error arising from this source, however, is opposite in direction to that introduced by hydrolytic breakdown.

Monoiodotyrosine Content of Thyroid Hydrolysates—Monoiodotyrosine comprises approximately 15 per cent of the total iodine in the rat thyroid hydrolysate (Table IV) and apparently somewhat more in the bird thyroid hydrolysate. This value is subject to the errors mentioned above but is probably accurate within 10 per cent. Its reliability is enhanced by the finding that the use of butanol-acetic acid-water instead of collidine-water-ammonia gave almost the same results (Table V). The values therefore could not have arisen simply as solvent artifacts.

It should be noted that the specific activity of the monoiodotyrosine iodine shortly after the injection of I^{131} is higher than that of any other fraction appearing on the chromatogram. This was confirmed in many separate experiments (Table VI). These data emphasize that the monoiodotyrosine could not have arisen from the breakdown of thyroxine or diiodotyrosine during the prolonged alkaline hydrolysis.

Diiodotyrosine Content of Thyroid Hydrolysates—Diiodotyrosine iodine

² It is of interest to record here the chromatogram of a sample of I^{131} obtained from Oak Ridge. The original I^{131} solution, diluted appropriately with 0.01 N NaOH and chromatographed in collidine- NH_3 , gave a single intense spot and one or two much weaker spots. In three experiments, 98, 85, and 97 per cent of the added I^{131} were recovered in the inorganic spot, as determined by cutting up the filter paper and eluting the various sections with 0.01 N NaOH.

TABLE IV
Iodine Content of Various Fractions of Thyroid As Separated by Filter Paper Chromatography

Section of chromatogram (see Fig. 1)	Component represented	Thyroids of 4 rats* 10 min. after injection with ^{131}I ; hydrolyzed with 2 N NaOH			Thyroids of 4 rats† 48 hrs. after injection with ^{131}I ; hydrolyzed with 2 N NaOH			Thyroids of 1 domestic fowl 24 hrs. after injection with ^{131}I ; hydrolyzed with 2 N NaOH			Sheep thyroids (see text for method)	
		Iodine	Per cent of total iodine	Specific activity	Iodine	Per cent of total iodine	Specific activity $\times 10^{-1}$	Iodine	Per cent of total iodine	Specific activity $\times 10^{-1}$	Iodine	Per cent of total iodine
1	Diiodotyrosine	γ	12	315	γ	11	163	γ	10	450	γ	14
2		9.0	27	371	2.9	10	539	4.4	24	413	16.9	31
3		19.1	17	469	9.3	33	584	10.3	22	427	37.4	18
4		12.0	9	230	4.5	16	727	9.6	8.6	246	21.9	5
5	Thyroxine	6.5	19	244	1.2	4.2	546	3.7	22	334	6.1	18
6		13.5	20	255	5.2	18	316	9.3	14	316	21.9	13
7		14.2	8	121	5.9	21	264	6.2	15	106	15.8	3
		5.6			3.0	11	159	6.3			3.9	
Sum, Sections 1-7.....		79.9	112		32.0	113		49.8	116		123.9	102
Total determined by analysis.....		72.0			28.4			43.0			121	
Trichloroacetic acid-soluble iodine.....		6.0		1139	4.5		2320					

* Rats of Curtis-Dunning strain raised on diet containing $2.5\text{ }\mu\text{Ci}$ of ^{131}I per gm.

† Rats of Long-Evans strain raised on diet containing $1\text{ }\mu\text{Ci}$ of ^{131}I per gm.

represents approximately 30 per cent of the iodine of the rat thyroid hydrolysate and about 25 per cent of the bird thyroid hydrolysate. In this case, also, the reliability of the results is supported by the finding that, when butanol-acetic acid-water was used as solvent, the results were similar to those obtained when collidine-water-ammonia was used as solvent. In previous methods (6, 7, 9, 10) used for the fractionation of

TABLE V

Comparison of Results Obtained with Collidine-Water-Ammonia and with Butanol-Acetic Acid-Water in Filter Paper Fractionation of Rat Thyroids

Sample No.	Per cent of total iodine in							
	Monoiodotyrosine fraction		Diiodotyrosine fraction		Thyroxine fraction		Inorganic iodide fraction	
	Collidine-water-NH ₃	Butanol-acetic acid-water	Collidine-water-NH ₃	Butanol-acetic acid-water	Collidine-water-NH ₃	Butanol-acetic acid-water	Collidine-water-NH ₃	Butanol-acetic acid-water*
1	18	16	25	27	19	38	20	13
2	15	15	26	28	16	30	23	17
3	14	14	26	30	19	41	20	14
4	15	13	30	27	17	47	19	7.5

* These values are low because of the loss of iodide as volatile HI during the chromatography with the acid solvent.

TABLE VI

Specific Activities (Counts per Second per Microgram of Iodine) of Iodine Fractions of Rat Thyroid 10 Minutes after Injection of I¹³¹

Experiment No.	Section 1	Section 2, diiodotyrosine	Section 3, monoiodotyrosine	Section 4	Section 5, thyroxine	Section 6, inorganic	Section 7	Trichloro-acetic acid-soluble, inorganic
1	192	186	252	157	99	99	101	1210
2	184	238	292	125	101	106	78	947
3	180	244	322	137	119	110	140	1090
4	121	252	332	140	116	111	81	1030
5	470	755	821	394	354	320	202	3260
6	514	552	794	333	324	311	194	1388

thyroid iodine, the diiodotyrosine fraction would most probably have included monoiodotyrosine.

Thyroxine Content of Thyroid Hydrolysates—Thyroxine iodine amounted to 15 to 22 per cent of the total iodine in the thyroid hydrolysate when the chromatogram was prepared with collidine-water-NH₃. When butanol-acetic acid-water was used as the solvent, however, the thyroxine values amounted to 30 to 47 per cent. The latter values are probably not trust-

worthy, because the thyroxine band was almost coincident with the solvent front and could therefore have been contaminated with other iodine-containing components. The values obtained with the collidine solvent, on the other hand, may be too low because of the breakdown of thyroxine which occurred during the chromatography itself. Evidence for the breakdown of thyroxine during chromatography with the collidine-ammonia solvent is presented in the following report dealing with the nature of plasma iodine (11).

It must be remembered that labile inorganic iodide was removed from the thyroid tissue before hydrolysis by treatment with trichloroacetic acid. Most of the inorganic iodide which appeared on the chromatograms, therefore, must have arisen from the breakdown of organic iodine during the prolonged alkaline hydrolysis (Table IV). Some may also have resulted from the breakdown of organic iodine, especially thyroxine, during the chromatography in collidine-water-NH₃. The values for inorganic iodide in Section 6 of the chromatogram (see Table IV), therefore, have no physiological significance.

The physiologically significant inorganic iodide of the gland is the fraction soluble in trichloroacetic acid (Table IV). In the rat thyroid, this fraction usually amounted to less than 10 per cent of the total iodine. Its specific activity was much higher than that of any other fraction 10 minutes after injection of I¹³¹. This observation is of particular interest in view of the report of Mann *et al.* (12) that the specific activity of the inorganic iodide fraction of the dog thyroid at an early interval after injection of I¹³¹ was *lower* than that of the diiodotyrosine-like fraction. This led these workers to postulate that the diiodotyrosine of the thyroid is formed at the level of the cell membrane. The data reported here fail to support such a view.

All the I¹³¹ in the trichloroacetic acid-soluble fraction of the thyroids of rats injected 10 minutes was shown to be inorganic by the finding that it was completely extractable with CCl₄ after the addition of iodide carrier and excess iodate.

The position of the inorganic iodide fraction on the chromatogram prepared with the collidine-water-NH₃ solvent (R_F approximately 0.7) is of particular interest. This implies, according to the partition theory of filter paper chromatography (13), that inorganic iodide favors the mobile phase (collidine) more than it does the aqueous phase. That this is indeed the case was shown by shaking 3 γ of labeled iodide with a mixture of 25 cc. of collidine and 25 cc. of 0.6 N NH₄OH. At equilibrium, the iodide concentration in the collidine phase was 2.7 times that in the aqueous phase.

Is 3,5-Diiodothyronine a Component of Thyroid Tissue?—Crystalline 3,5-diiodothyronine was added to a hydrolysate prepared from the thyroid

of rats injected with I^{131} , and the chromatogram was prepared, as described above, with collidine-water- NH_3 . The position of diiodothyronine, as revealed by spraying with diazotized sulfanilic acid was just below that of inorganic iodide (R_F approximately 0.65). Two different preparations of 3,5-diiodothyronine were tested and these gave identical results. There was no radioactive band on the autograph corresponding to this position. This experiment rules out 3,5-diiodothyronine as an intermediate in the formation of thyroxine in the rat thyroid. It should be recognized, however, that any iodine compound in the thyroid that does not become appreciably labeled upon injection of I^{131} would not be detected by the autographic procedure used here.

3',5'-Diiodothyronine was not available for testing.

Fractionation of Iodine in Sheep Thyroid Hydrolysate—It was not convenient to inject radioactive iodine into large animals such as sheep. The following indirect method was therefore used for labeling the various iodine components of the sheep thyroid gland. Thyroid glands were obtained from two freshly killed lambs at the local slaughter-house and brought to the laboratory packed in ice. They were trimmed carefully and minced with scissors. Weighed portions of these glands were then labeled by the addition of one lobe of the thyroid of a rat which had been maintained on a low iodine diet and injected 3 hours previously with 150 $\mu c.$ of I^{131} . A rat lobe weighing 20 mg. was added to 196 mg. of sheep gland; a rat lobe weighing 17.2 mg. was added to 159 mg. of another sheep gland. The mixed tissues were ground with 1 cc. of 10 per cent trichloroacetic acid and the washed precipitates were hydrolyzed with 1.5 cc. of 2 N NaOH. The hydrolysates were extracted with butanol and chromatograms were prepared by use of collidine-water-ammonia solvent. Radioautographs of these chromatograms showed the usual pattern, and the chromatograms were cut up for iodine analysis as described above. The distribution of the iodine in the sheep gland, as determined with this procedure, is shown in Table IV. The contribution of the rat thyroid lobe to the total iodine is very small because (1) it represented only about one-tenth of the total thyroid tissue, and (2) it contained only about 10 mg. per cent of total iodine (the rat had been maintained on a low iodine diet). The sheep gland, however, contained 75 mg. per cent of total iodine.

The results shown in Table IV indicate that sheep thyroid also contains moniodotyrosine and that the distribution in this animal of iodine among the various fractions studied is similar to that of the bird and rat.

DISCUSSION

In 1939, Ludwig and von Mutzenbecher (14) isolated a crystalline compound from the hydrolysis products of iodinated casein which they believed to be moniodotyrosine. It was isolated from the acid-soluble

iodine fraction and separated from diiodotyrosine by fractional crystallization. The identity of this compound was established by elementary analysis and by the fact that it gave 3,5-diiodotyrosine on treatment with iodine, and tyrosine on catalytic deiodination.

Subsequently, Herriott (15) reported the isolation of crystalline moniodotyrosine from partially iodinated pepsin. His description of the isolated compound differed in several respects from that given by Ludwig and von Mutzenbecher, and the identity of the compound has been questioned by Harington and Pitt-Rivers (16). The latter workers synthesized both 3-monoiodo-L-tyrosine and 3-monoiodo-DL-tyrosine and found that neither corresponded exactly in properties with the compound isolated by Herriott, but that their monoiodo-DL-tyrosine did agree with the compound described by Ludwig and von Mutzenbecher. The monoiodo-DL-tyrosine synthesized by Harington and Pitt-Rivers was the source of that material used in the present investigation.

Herriott later reinvestigated the nature of the iodine compound isolated from partially iodinated pepsin (17). After repeated recrystallization, this product was found to be identical with synthetic 3-monoiodo-DL-tyrosine with respect to ultraviolet absorption spectra, pK value of the phenol group, solubility measurements, and partition coefficients. Herriott estimated that 73 per cent of the iodine in an acid hydrolysate of partially iodinated pepsin is in the form of 3-monoiodotyrosine.

In 1947, Roche and Michel (18) described a procedure for the colorimetric microdetermination of thyroxine, diiodotyrosine, moniodotyrosine, and tyrosine in iodinated proteins. Their method depends on the fact that only thyroxine and diiodotyrosine give the color reaction, with nitrous acid, characteristic of ortho diiodophenols, whereas only moniodotyrosine and tyrosine give the Millon test. The spectral characteristics of the color given by moniodotyrosine with Millon's reagent are only slightly different from those of the tyrosine derivative, and the determination of one in the presence of the other involves the determination of extinction coefficients at two different wave-lengths. Although the French investigators found quite appreciable quantities of moniodotyrosine in artificially iodinated protein (19) and in many species of coral (20), they reported that thyroglobulin was almost devoid of this component (18).

It can hardly be doubted, from the results presented here, that there exists, in hydrolysates of both rat and chicken thyroid, a significant organic iodine component (not thyroxine or diiodotyrosine) which amounts to 15 to 20 per cent of the total iodine. This component has been identified as moniodotyrosine because its behavior is identical with that of added crystalline moniodotyrosine, as judged by filter paper partition chromatography. This identity in behavior is observed under two entirely different sets of conditions: (1) when a solvent mixture consisting of the or-

ganic base collidine and water is allowed to move through the paper in an atmosphere of ammonia, and (2) when a mixture of butanol, acetic acid, and water is used as the solvent.

Is Monoiodotyrosine a Normal Constituent in Thyroid Gland?—The possibility that the monoiodotyrosine arises during the fairly drastic hydrolysis of the thyroid protein was not completely excluded by previous investigators (4, 21). It has, however, been ruled out in the present investigation by two separate lines of evidence: (1) when known quantities of crystalline diiodotyrosine or thyroxine were added to rat thyroid tissue before hydrolysis, only 2 to 3 per cent of the added iodine appeared in the monoiodotyrosine fraction (Table III); (2) the iodine in the monoiodotyrosine fraction has the highest specific activity of any of the iodine fractions in a thyroid hydrolysate at a very early interval after I^{131} injection (Table VI). These findings, especially the latter, indicate that monoiodotyrosine is not only a real component of the thyroid, but is also very likely a precursor of diiodotyrosine in the formation of thyroxine. A similar conclusion was reached by Roche *et al.* (19) with regard to the significance of monoiodotyrosine in the formation of thyroxine in iodinated casein.

A great many autographs similar to those shown in Fig. 1 have been prepared in this laboratory and in every case the same four major components have appeared. A fifth component, just above the origin (Section 1), frequently appears when the collidine- NH_3 solvent is used. The significance of this component has not been established. It may represent incompletely hydrolyzed thyroglobulin.

The large fraction of inorganic iodide found in the hydrolysate indicates that a good deal of breakdown occurs during the hydrolysis. Even when 8 per cent $Ba(OH)_2$ is used as the hydrolytic agent, the inorganic iodide fraction of the chromatogram amounts to 20 per cent of the total iodine. It is not surprising, therefore, that appreciable quantities of iodine are found in the sections of the chromatogram (Sections 1, 4, and 7) that do not correspond to known iodine components of the gland. Whether all the iodine in these sections represents breakdown products arising during hydrolysis or chromatography, or whether some of this iodine represents unknown iodine components of the gland, cannot be decided at the present time. The problem of determining all the iodine-containing components of the thyroid gland must await better methods of hydrolysis. Enzymatic hydrolysis would seem to offer the most promise for releasing these components from peptide linkage without excessive breakdown.

We wish to express our appreciation to Sir Charles Harington for supplying the monoiodotyrosine used in this investigation, and to Dr. H. Sidney Newcomer of E. R. Squibb and Sons for a supply of thyroxine.

SUMMARY

1. A technique for the quantitative fractionation of the iodine-containing components of a thyroid hydrolysate by filter paper partition chromatography is described.

2. The presence of moniodotyrosine, in addition to diiodotyrosine and thyroxine, in the thyroid of the rat, chicken, and sheep is established.

3. Hydrolysis of thyroid tissue with either 2 N NaOH or 8 per cent Ba(OH)₂ leads to fairly extensive breakdown of thyroxine.

4. Moniodotyrosine comprises approximately 15 per cent of the total iodine in the hydrolysate of rat thyroid and approximately 20 per cent in that of chicken thyroid.

5. Diiodotyrosine comprises approximately 30 per cent of the total iodine in the hydrolysate of rat gland and about 25 per cent in that of chicken gland.

6. The method described here does not appear to be suitable for the quantitative estimation of thyroxine. It may be said, however, that the latter component comprises at least 20 per cent, and possibly much more, of the total iodine in the hydrolysate.

7. 3,5-Diiodothyronine appears to be absent from the rat thyroid.

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THE NATURE OF PLASMA IODINE AS REVEALED BY FILTER PAPER PARTITION CHROMATOGRAPHY*

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Despite the fact that crystalline thyroxine completely replaces the function of the thyroid gland in thyroidectomized animals, it is not universally accepted that thyroxine itself is the actual form of the circulating thyroid hormone. The reasons for this have been pointed out in a previous report from this laboratory (1). In that report, experimental evidence was also presented favoring the view that the greatest part of the so called "protein-bound iodine" of plasma is actually thyroxine loosely attached to protein.

The sensitivity of the technique of filter paper partition chromatography, as described in the preceding paper, suggested a new approach to a study on the nature of plasma iodine. The application of this technique to the fractionation of plasma iodine is described here.

EXPERIMENTAL

Rats which had been raised on a fairly low iodine diet (0.3 γ per gm.) were injected with 150 μ c. of carrier-free I^{131} . 24 hours later, blood was removed by heart puncture and pooled, and the plasma separated. It has been shown previously in this laboratory that plasma I^{131} at that interval is largely in the form of protein-bound iodine (2). When a portion of the pooled plasma was treated with 10 per cent trichloroacetic acid, 85 to 90 per cent of the total radioactivity was precipitated with the proteins. It may therefore be concluded that only about 10 to 15 per cent of the radioiodine in this plasma was in the form of inorganic iodide.

Butyl alcohol extracts were prepared from 4 cc. portions of the radioactive plasma. Each 4 cc. portion was extracted with 10 cc. of butyl alcohol in 40 cc., narrow necked, round bottom centrifuge tubes. The layers were separated by centrifugation, and the clear butanol layer was drawn off. The residue was extracted a second time with 10 cc. of butanol, and the clear butanol layer obtained by centrifugation was combined with the first extract. The final butanol extract contained about 70 per cent of the total radioactive iodine.

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The butanol solutions were delivered to filter paper strips exactly as described in the previous paper (3). However, since the volumes of butanol were larger (usually 22 cc.) and the amounts of radioactivity contained therein were considerably less than in the case of the thyroid extracts, it was necessary to transfer more of the solution to the filter paper. A total of 500 μ l. was delivered in 25 μ l. portions. The chromatograms were prepared with the collidine-water-NH₃ solvent as previously described. Chromatograms were also prepared from the *same* butanol extract to which had been added an appropriate amount of crystalline thyroxine in 0.1 cc. of weakly alkaline solution. This provided 30 to 40 γ of thyroxine in the butanol extract delivered to the paper. These chromatograms were sprayed with the diazotized sulfanilic acid reagent to locate the added thyroxine.

Radioautographs were prepared by exposing the dried chromatograms to no screen x-ray film for 5 days.

Results

The radioautographs of the chromatograms are shown in Figs. 1 and 2. Fig. 1 is that of the untreated butanol extract, Fig. 2 that of the same butanol extract to which thyroxine had been added. *The intense band seen in Fig. 2 ($R_F = 0.47$) coincided exactly, both in shape and in position, with the added thyroxine as determined by the color test.* The fact that this band was somewhat different in shape from that in Fig. 1 may be attributed to the tremendous difference in the quantity of thyroxine present in the two cases. In the case of the butanol extract containing added thyroxine, the quantity of I¹²⁷ delivered to the paper was approximately 30 γ . In the case of the untreated butanol, the amount of iodine delivered to the paper was only about 0.004 γ , an amount below the limits of sensitivity of the micromethods now available for measurement of iodine. This is an excellent demonstration of the sensitivity of the chromatographic technique.

In one experiment the filter paper chromatograms were divided into sections as shown in Fig. 1. The radioiodine in each section was then determined by digesting the filter paper with chromic acid, distilling the iodine, and counting it with a sensitive mica window counting tube. 2 γ of iodide carrier were added before the digestion to improve the recovery of radioiodine in the distillate.

The recoveries of I¹³¹ in the various sections of the chromatogram are shown in Table I for three different conditions: (1) the untreated butanol extract of plasma, (2) the butanol containing added thyroxine, and (3) the butanol extract containing added diiodotyrosine. In all cases, the largest fraction of the added radioiodine was recovered in the thyroxine

section. When thyroxine carrier was added, 75 per cent of the radioiodine appeared in the thyroxine section, but in the untreated butanol extract, or when diiodotyrosine carrier was added, only 55 per cent was recovered in this section. At the same time, only 16 per cent of the radioiodine was found in the *inorganic* section when thyroxine carrier was added, whereas 24 per cent appeared in this section in the case of the untreated butanol

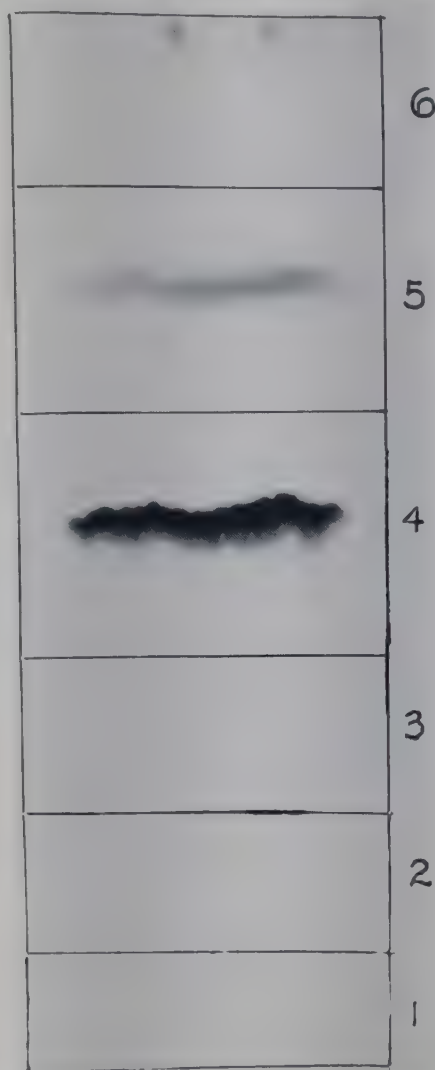


FIG. 1. Radioautogram of ascending filter paper chromatogram prepared from a butanol extract of the plasma of rats injected for 24 hours with I^{131} . Exposure time 5 days.

extract. The latter figure is considerably higher than that found in the trichloroacetic acid-soluble fraction of this plasma (13 per cent).

These findings may be explained on the assumption that considerable breakdown of thyroxine (or whatever the major organic iodine component of the plasma is) occurs during the chromatography, and that this breakdown is proportionately less when larger amounts of thyroxine are present.

The possibility of exchange between radioactive inorganic iodide and added thyroxine must also be considered. That this may not be appre-



FIG. 2. Left, ascending filter paper chromatogram of same butanol extract as that used in Fig. 1, but containing added thyroxine. The position taken by the added thyroxine was determined by spraying with diazotized sulfanilic acid. Right, the radioautogram of this filter paper. Note the correspondence in position and shape between the intense spot in the radioautogram and the line drawing of the thyroxine in the chromatogram.

ciable, however, is suggested by the absence of any exchange between inorganic iodide and added diiodotyrosine (Table I).

In several experiments, the butanol extracts were reextracted with 4 $\times$ NaOH-5 per cent Na_2CO_3 before being applied to the filter paper. This reagent removes inorganic iodide and diiodotyrosine, but not thyroxine.

from a butanol solution. It was expected, in this case, that the autograph would show only a single band, that corresponding to thyroxine. However, the autograph prepared from the chromatogram of this reextracted butanol showed not one, but three, bands. In addition to that of thyroxine, the bands corresponding to inorganic iodine and diiodotyrosine were very prominent. Apparently a good deal of breakdown of organic iodine occurs when the highly alkaline butanol extract is applied to the paper, or during its chromatography with collidine-NH₃.

TABLE I

Filter Paper Partition Chromatography of Butanol Extract of Plasma Obtained from I¹³¹-Injected Rats

Section of chromatogram (see Fig. 1)	Component represented	Recovery of added radioactive iodine					
		Untreated extract		28.4 γ thyroxine iodine added		10.1 γ diio- dotyrosine iodine added	
		Counts per sec.	Per cent of total	Counts per sec.	Per cent of total	Counts per sec.	Per cent of total
1	Origin	2.4	1.5	2.0	1.2	2.4	1.4
2	Diiodotyrosine	6.4	3.9	4.4	2.6	6.8	4.0
3		4.0	2.4	5.2	3.1	5.2	3.0
4	Thyroxine	90.0	55	125.6	75	91.6	54
5	Inorganic	39.2	24	27.2	16	48.0	28
6		21.2	13	9.6	5.7	0	0
Total, Sections 1-6.....		163		174		154	
" added to paper....		164		167		171	

Comment

A previous report (1) points out that the very minuteness of the concentration of iodine in plasma made its chemical identification difficult. By employing the radioactive isotope of iodine, I¹³¹, together with a refined method for determining small quantities of iodine, we were, however, able to provide evidence that circulating hormonal iodine is actually thyroxine. In the present investigation, the nature of plasma iodine was further studied by means of filter paper partition chromatography. The sensitivity of this new procedure permitted us to identify the iodine equivalent of 0.1 cc. of plasma, or approximately 0.004 γ of iodine.

A butanol extract of the plasma of rats injected with I¹³¹ shows only two major I¹³¹-containing components when examined by filter paper partition chromatography with collidine-NH₃. One of these corresponds exactly to the position taken up by added thyroxine, and the other, to the

position taken up by added inorganic I^{131} . In some experiments, a faint band corresponding to diiodotyrosine also appeared, as did a faint darkening at the solvent front.

These results support the view, set forth in a previous communication from this laboratory (1), that the circulating thyroid hormone consists of thyroxine loosely attached to plasma protein. However, it is not certain that the procedure used here could be used to distinguish thyroxine from one of its peptides. The possibility, therefore, that thyroxine circulates in the organism as a small peptide has not been ruled out. A further study, with synthetic small peptides of thyroxine, should help resolve this problem.

SUMMARY

1. Plasma obtained from rats that had been injected 24 hours earlier with I^{131} was extracted with butanol, and the nature of the organic I^{131} -containing components of the extract was examined by filter paper chromatography.

2. The position taken by the major radioactive component, as determined by radioautographs of the filter paper, corresponded exactly to that of thyroxine carrier as determined by color test. The organic iodine of plasma, therefore, appears to be identical with thyroxine.

Addendum—After this manuscript had been submitted there came to our attention a report by Laidlaw (4), who also employed filter paper partition chromatography to study the nature of the circulating thyroid hormone. His conclusions are essentially in agreement with those presented here.

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EFFECT OF SODIUM CITRATE AND HEPARIN ON REMOVAL OF CALCIUM FROM BLOOD AND SERUM BY AMBERLITE*

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It was observed by one of us (F. S.) that Amberlite IR-100 failed to remove calcium quantitatively from citrated plasma (obtained from a blood bank). Since Steinberg (1) and later Quick (2) had found that this resin completely removed calcium from blood, it appeared probable that sodium citrate interfered with the adsorption of calcium by Amberlite, especially since citrate forms a complex with calcium, thereby depressing its ionization. A quantitative study was therefore made to correlate the adsorption of calcium by Amberlite with increasing concentrations of sodium citrate.

EXPERIMENTAL

Collection of Serum—Blood obtained by venipuncture from healthy young adults was allowed to clot at room temperature. The various sera were separated by centrifugation and pooled.

Preparation of Amberlite—Amberlite IR-100, analytical grade, was purified according to the method of one of us (3) and charged in the sodium cycle. Preliminary studies indicated that 0.5 gm. of the resin would completely decalcify 1 ml. of serum.

Determination of Calcium—In all cases serum calcium was determined by the method of Clark and Collip (4).

Removal of Calcium by Amberlite As Influenced by Sodium Citrate and Heparin—In one series of experiments serum was passed three times through a column of Amberlite according to the method previously described (3). Usually 5 ml. of serum were passed through 2.5 gm. of Amberlite. In another series 10 ml. of serum were passed through 5 gm. of Amberlite, which was contained in a Chohnoky column, by gravity and finally suction. In control experiments a 5 ml. solution of CaCl_2 in physiological saline, containing 10 mg. of Ca^{++} in 100 ml. (27.693 mg. of anhy-

* The opinions expressed in this paper are those of the authors, and do not necessarily represent the official views of any governmental agency.

drous CaCl_2 in 100 ml. of 0.85 NaCl solution), was passed through Amberlite (2.5 gm.) under the same conditions.

To study the effect of sodium citrate on the adsorption of calcium by Amberlite, 1 ml. of different molarities of sodium citrate was added to 9 ml. of pooled serum or standard CaCl_2 solution. The concentration of calcium was first determined in the samples. Then the aliquots were passed through the column of Amberlite. After treatment of the samples

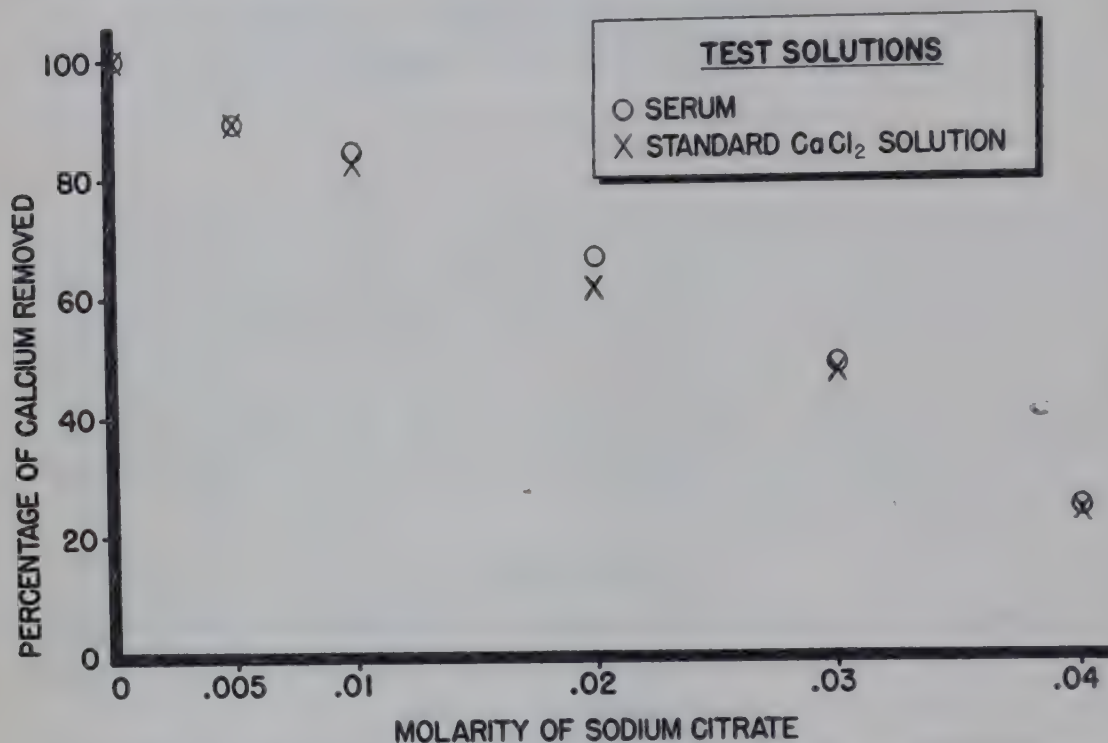


FIG. 1. Effect of varying concentrations of sodium citrate on the amount of calcium removed by Amberlite. The calcium concentrations have been expressed as percentage of calcium removed. Sodium citrate has been expressed in moles. In each case 1 ml. of sodium citrate solution was added to 9 ml. of test solution. O represents the values obtained with serum; X those obtained with a standard CaCl_2 solution.

the calcium concentration was again determined. The final results were expressed as the percentage of calcium originally present which was adsorbed by Amberlite.

To study the influence of heparin on the adsorption of calcium by Amberlite, blood was obtained by venipuncture and drawn into a siliconized syringe. This whole blood was placed in tubes containing 1 to 4 γ of heparin per ml., thoroughly mixed, and immediately passed through a column of Amberlite, as previously described (3).

Results

Effect of Sodium Citrate on Adsorption of Calcium by Amberlite—Amberlite removed calcium so completely from normal serum that none could be detected, by the method employed, after treatment with this resin. On addition of sodium citrate to serum, the removal became incomplete. The findings are summarized in Fig. 1. Two points stand out: (1) An inverse, roughly linear relationship appears to exist, over the range studied, between the molarity of sodium citrate and the amount of calcium adsorbed by Amberlite. (2) This relationship holds whether serum or a solution of calcium chloride is used.

Effect of Heparin on Adsorption of Calcium by Amberlite—Calcium can be completely removed from heparinized plasma or serum.

DISCUSSION

Sodium citrate forms a complex with calcium, apparently so stable that the adsorption of this calcium by Amberlite is drastically depressed. Since Amberlite removes calcium quantitatively from blood, it can be concluded that the union of calcium with the proteins and possibly other constituents of the plasma is, in contrast, sufficiently tenuous to be disrupted by Amberlite.

It should be noted that when 1 volume of 0.1 M sodium citrate is added to 9 volumes of serum or plasma, a concentration of sodium citrate which is sufficient to inhibit coagulation completely, 84 per cent of the calcium is still removable by Amberlite. This suggests that when sodium citrate is added to serum or plasma to bring the concentration to 0.01 M an appreciable amount of calcium remains ionized. This finding is in accord with the observations of Hastings *et al.* (5) that the primary dissociation constant of calcium citrate is relatively high ($pK_{CaCit} = 3.22$). Sabbatani (6) postulated that the anticoagulant action of sodium citrate was due to the depression of calcium ions. His explanation has been generally accepted, but Quick and Stefanini (7) have recently presented evidence that the primary anticoagulant action of sodium citrate is antiprothrombic; *i.e.*, the citrate combines with prothrombin, thereby inactivating it. This action occurs at a citrate concentration at which considerable ionized calcium is still present. The new observation, that most of the calcium is still removable at a concentration of sodium citrate which completely inhibits coagulation, is in accord with the view that the primary anticoagulant action of sodium citrate is not due to a depression of the calcium ion concentration. Only at higher concentrations of sodium citrate is the depression of calcium ions sufficiently great to become a secondary factor in the inhibition of coagulation.

SUMMARY

1. A study was made of the influence of heparin and sodium citrate on the adsorption of calcium by Amberlite IR-100 in the sodium cycle. The calcium solutions tested were plasma, serum, and a standard calcium chloride solution.

2. An inverse, roughly linear relationship was found between the molarity of sodium citrate and the amount of calcium removed from the several solutions tested.

3. Heparin did not interfere with the decalcifying action of Amberlite.

4. The implication of these results in regard to coagulation is discussed.

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CARBONIC ANHYDRASE IN THE BRAIN OF THE NEW-BORN IN RELATION TO FUNCTIONAL MATURITY*

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From studies of the carbonic anhydrase content of the central nervous system of fetal cattle and of premature human infants, it was previously reported that this enzyme first appears in greater amounts in the lower centers and that the maximum amount progresses to higher centers as the fetus develops. The enzyme was not found in the cerebrum of fetal cattle until the last month of gestation, during which it rapidly rose to an amount equal to half of that of the adult animal. Although a similar progressive increase in the enzyme was shown in the human fetus, as studied in premature births, none was found in the cerebrum at 8 months or in the cerebrum of an 8 pound, full term infant. Although this child was well developed, the cause of death was not evident. The normality of this finding, for the central nervous system, was therefore questioned (1).

With the purpose of clarifying this finding in a human infant, a series of studies was made upon the young of animals of different degrees of maturity at birth. The dog, the rat, the cat, and the rabbit were chosen as examples of animals less mature at birth, and the guinea pig as an example of an animal more mature at birth, than the human infant. Opportunity was also offered to study the central nervous system of a second human new-born which failed to survive, owing to a well defined cause not associated with the central nervous system.

Technique

The method used in determining carbonic anhydrase was a variant of the procedure of Philpots and Philpots, modified by Keilin and Mann. This has been described (2). A Hoke-Phoenix valve and a Fisher gas-flow indicator containing mercury were used to stabilize the flow of carbon dioxide.

Rats from several litters were studied at seventeen different ages, ranging from birth to 40 days. For comparison, averages of findings in the cerebellum and cerebrum were made on fourteen older rats that had been used as controls in another investigation. For a determination in

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the new-born, the brains of eight animals were required. As the animals grew larger and the amount of carbonic anhydrase in the material increased, it was possible to make determinations separately for the cerebrum and cerebellum plus the brain stem on individuals.

The puppies were of two litters. The first litter of four was from a mongrel fox terrier, and the second litter of five from a mongrel beagle hound. The puppies were sacrificed at birth and at intervals up to 36 days. Comparison was made with the findings in adult dogs sacrificed at the city pound. Determinations were made on the cord, the medulla and pons combined, the cerebellum, the thalamus, the caudate nucleus, the cerebrum, and upon the packed red blood cells. In two dogs the enzyme content of the olfactory bulbs was measured.

Three kittens were similarly studied, except that the first animal of the series was 6 days old.

The three infant rabbits tested were 2 days old.

The human infant studied was a well developed child weighing 8 pounds, 3 ounces, which was born at 10 a.m. It died on its way to the hospital of multiple hemorrhages. Autopsy was performed at 2 p.m. on the same day. The brain weighed 430 gm. and was well developed.

Of the guinea pigs, one was born over the week-end and was less than 2 days old when studied. Two were taken from the mother late in pregnancy; their eyes were open, and they were 6 inches long and weighed 103 and 91 gm., respectively. Two were taken from the mother earlier in pregnancy; their eyes were closed; one was $3\frac{1}{2}$ inches long and weighed 21 gm., and the other was $3\frac{3}{4}$ inches long and weighed 24 gm. The two mothers were used as controls.

Results

In infant rats no carbonic anhydrase was found in the brain during the first 3 days after birth. Between the 5th and 12th days there was a very small amount of the enzyme found in the cerebrum, which did not appreciably change during this period, while the content of the cerebellum was greater and showed an increase. During this time the external orifice of the ears had developed but the eyes were closed. The opening of the eyes occurs between the 14th and 17th days after birth. By the 14th day a steady increase in carbonic anhydrase in the cerebrum had started. This increase appears to continue beyond 40 days, as the average content of fourteen adult rats was greater than that of any animals 40 days old or younger. The cerebellum plus the brain stem, on the other hand, showed a measurable content at 3 days and had attained an adult level by 30 days. The changes in the carbonic anhydrase content of the rat brain are charted in Fig. 1.

The first puppies were sacrificed a few hours after birth, at which time a considerable amount of the enzyme was found in the blood. The carbonic anhydrase as determined on the packed corpuscles of one of the new-born puppies was found to equal 40 per cent of that found in a 36 day-old puppy. In spite of this considerable amount of the enzyme in the blood of the new-born, none was found in the central nervous system of either puppy, unless there may have been a trace in the pons and medulla of the terrier pup. Owing to the technical error involved in testing tissue of low carbonic anhydrase content, but which contains blood having a high content of the enzyme, some of these determinations in the new-born

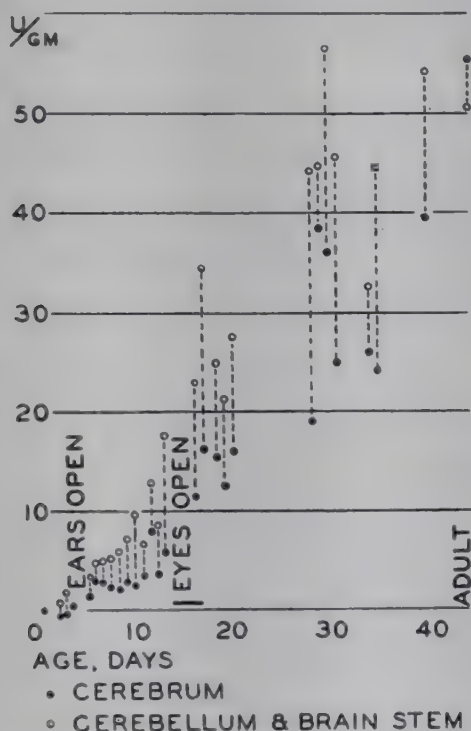


FIG. 1. Change in carbonic anhydrase content of the rat brain with development.

animals gave a small negative quantity. By the 8th day the terrier pup, whose eyes were still closed, showed a measurable amount of carbonic anhydrase in the cerebellum, the pons-medulla, and the cord, but there was still no evidence of the enzyme in the cerebrum. The cerebrum of the 9 day-old beagle pup, whose eyes were open, probably contained a small amount of carbonic anhydrase, as a positive quantity was obtained on three determinations. There was some evidence of carbonic anhydrase in the rostral half of the cerebrum of the 15 day-old terrier pup whose eyes had been open 1 day, but none was found at the occipital pole. By the time the puppies were 29 and 36 days old and were barking and showing

curiosity, the carbonic anhydrase in the cerebrum averaged a fifth of that found in the adult dog brain. The olfactory bulbs of two of the larger

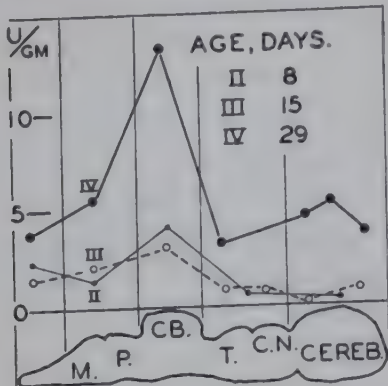


FIG. 2

FIG. 2. Carbonic anhydrase found in the central nervous system of fox terrier litter mates at different ages. M. = medulla, P. = pons, CB. = cerebellum, T. = thalamus, C. N. = caudate nucleus.

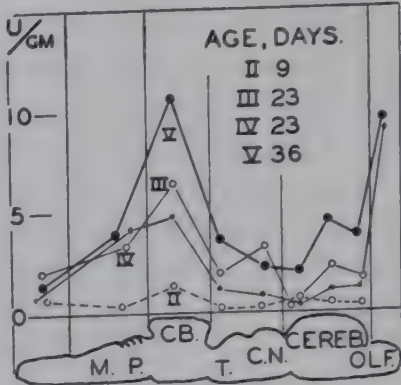


FIG. 3

FIG. 3. Carbonic anhydrase in the central nervous system of beagle hound litter mates. M. = medulla, P. = pons, CB. = cerebellum, T. = thalamus, C. N. = caudate nucleus, OLF. = olfactory bulb.

TABLE I

Increase in Carbonic Anhydrase Content of Central Nervous System of Cat with Development

The values are expressed as carbonic anhydrase units per gm.

Age, days.....	6	12	20	Adult	Adult
Degree of development.....	Eyes closed	Eyes open, no teeth	Teeth erupted		
Weight, gm.....	213	266	389		
Cerebrum, frontal.....	1.2	2.7	7.2	46.4	43.9
“ middle.....		3.2	8.3	54.0	
“ occipital.....	-0.2	1.9	4.2	66.0	50.9
Thalamus.....	5.0	2.2	4.2	26.4	34.2
Colliculi.....	5.4	2.8	4.3	21.7	
Cerebellum.....	3.3	2.6	8.0	41.7	
Pons.....				29.5	25.7
Medulla.....	5.3	3.1	6.8	30.6	31.1
Spinal cord.....	7.2	7.5	9.7	13.9	18.1 (Cervical cord)

pups were tested separately. Their content was very high when compared with the cerebrum. These results are charted in Figs. 2 and 3.

Three 2 day-old rabbits, whose brains were pooled, showed a low carbonic anhydrase content. The cerebrum contained 2.1 units per gm. and the cerebellum 3.3 units.

The data on the kittens are given in Table I. These values approximate the results from the puppies, except that carbonic anhydrase developed earlier in the lower centers of the brain.

The results from the lower centers of the brain of the new-born infant are practically identical with those already reported (1). In this child,

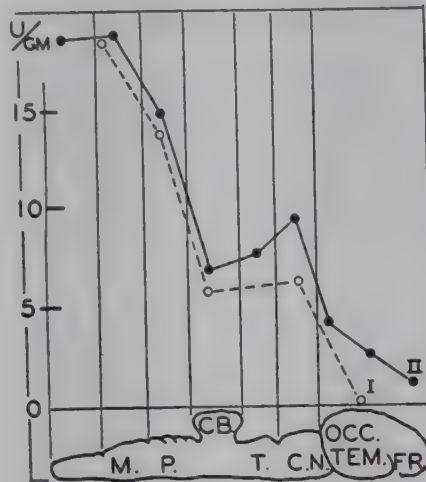


FIG. 4. Carbonic anhydrase content of the central nervous system of two new-born full term human infants. Infant I weighed 8 pounds at birth and died from an unknown cause. Infant II weighed 8 pounds, 3 ounces and died of hemorrhage. M. = medulla, P. = pons, CB. = cerebellum, T. = thalamus, C. N. = caudate nucleus, OCC. = occipital lobe, TEM. = temporal lobe, FR. = frontal lobe.

TABLE II

Carbonic Anhydrase in Central Nervous System of Developing Guinea Pig

The values are expressed as carbonic anhydrase units per gm.

Group	Degree of development	Cerebrum	Cerebellum	Cerebellum + brain stem	Brain stem
A	Fetus I, eyes closed.....		1.43, whole brain		
"	" II, " ".....		1.50, " "		
B	" I, " open.....	9.1		10.8	
"	" II, " ".....	8.6		15.7	
	New-born.....		15.5, whole brain		
A	Mother.....	18.8	20.5		8.6
B	".....	15.8	25.3		8.3

however, there was a small amount of the enzyme in the cerebrum. The contents of the frontal, temporal, and occipital poles were 1.0, 2.4, and 4.0 units per gm. respectively. The data are presented in Fig. 4 with those already reported from the child which weighed 3 ounces less at birth. A low carbonic anhydrase content for a normal adult would be 25 units per gm.

The brain of the new-born guinea pig contained an amount of carbonic anhydrase which approached that of the adult. The content of the fetal guinea pigs with open eyes was considerable; that for the younger fetuses, whose eyes were still closed, was very small. These data are given in Table II together with those obtained from the respective mothers.

DISCUSSION

The data on new-born puppies, cats, and rats indicate that it is normal for animals born while immature to have little or no carbonic anhydrase in the higher levels of the central nervous system at birth. In view of this, the finding previously reported (1) of no carbonic anhydrase in the brain of the human full term new-born probably represents a normal condition. To counterbalance the fact that there was some carbonic anhydrase found in the cerebrum of the second infant reported here, whose normality as to development was better established, we have the probability that this second child was of greater fetal age since there was very good development of the brain for a new-born child. The brain weighed 450 gm. as against 335 gm. for the average 7 pound new-born infant.

There appears to be a time correlation between the development of senses reporting more exclusively to the brain and the appearance of carbonic anhydrase in the cerebrum, which suggests the synchronization of the beginning of function of the brain with the appearance of the enzyme. In rats, carbonic anhydrase was absent from the brain at birth, rose to a low level with the opening of the ears, and remained there until shortly before the eyes opened at 14 to 17 days. It then increased gradually to adult proportions. In the two litters of puppies, carbonic anhydrase appeared in the cerebrum at the time of the opening of the eyes or soon after. In the case of the kittens the enzyme content increased to definitely measurable amounts after the eyes were opened. In two puppies in which the carbonic anhydrase content of the olfactory bulbs was tested, a comparatively high activity was found. This is of interest in view of the probability that puppies with unopened eyes find their way back to their mother through the sense of smell.

There also appears to be a relationship between the appearance of brain waves as reported in the literature and our findings on the development of carbonic anhydrase in the cerebrum. In the human infant, according to Gibbs and Gibbs (3), "The normal new-born infant shows irregular slow waves with a frequency of $\frac{1}{2}$ to 2 per second. . . No really steady frequencies are present and certainly there is nothing to correspond to the nine or ten-per-second waves so common in adults, which Berger calls alpha waves." By the time the child is 7 years old, the brain waves have almost reached the frequency found in maturity. In the brain of the new-born we found little or no carbonic anhydrase; in the brain of a

child of 7 we have found a carbonic anhydrase content comparable to an adult level. Unfortunately we have no intermediate findings. Jasper, Bridgeman, and Carmichael (4) made an ontogenetic study of the electrical potentials in the guinea pig. They obtained their controls by clamping off the maternal circulation. There was an onset of activity in the brain potentials 2 weeks before birth, at 48 days of gestation. The duration of bursts of activity increased with age. Three 56 day-old fetuses showed continuous brain potentials characteristic of the adult. The motor mechanisms of the eye were found to be well developed between 48 and 56 days of gestation and the eyes to be opened naturally. Changes in the character of the brain waves were found in response to stimulation on the 60th day of gestation.

Our findings for the carbonic anhydrase content of the new-born and fetal guinea pig would stand as a corollary to this work.

In the young rabbit, in which we found only traces of carbonic anhydrase at 2 days, Kornmueller (5) reports that there was only slight indication of electrical activity 6 days after birth and that complete development did not occur until 12 days after birth.

That there should be a relationship between function in an organism and increase in the presence of those enzymes taking part in this functioning is to be expected. In bacteria there is an increase in the enzyme specific for a substrate in response to the presence of the substrate in the culture medium. Boell (6) quotes Coghill (1929) and Sawyer (1943) in support of his own finding that the development of a high choline esterase level occurs with the functional maturation of the neuromuscular apparatus in *Amblystoma*. Youngstrom (7) found a similar relationship in the human fetus, and Nachmansohn in the goat (8). Moog (9) found a relationship between the rise of adenylypyrophosphatase activity in the brain, liver, heart, and muscle of chick embryos, which was more closely related to the onset of function than to growth. Mirski and Wertheimer (10) found extremely low phosphorylytic activity in the muscles of embryo rats and rabbits, which remained low until 3 days after birth, whereas preparations from heart and liver showed full phosphorylytic activity in these organs. These authors related the earlier appearance of the phosphorylytic enzyme in these organs to their earlier functioning. Palladin (11) reported that trained muscles showed an increased phosphocreatine content, and increase in succinic dehydrogenase and α -glycerophosphate dehydrogenase activity, incidental to an increase in respiratory processes. Meyer *et al.* (12) found a fluctuation in succinic dehydrogenase activity in rat ovaries with high and low lutein function. It would seem that, if enzymes which play a part in the essential metabolism of the organ increase with function, then conversely an enzyme of unknown place in the metabolism of the organ would probably play an essential part in function if it appeared upon

the onset of the functioning of the organ. The correlation of the appearance of carbonic anhydrase in the brain with the onset of brain waves, which are dependent upon aerobic metabolism, and the lack of carbonic anhydrase in the young rat, in which Fazekas *et al.* (13) find a preponderantly anaerobic metabolism, suggests that carbonic anhydrase may be an accessory to aerobic oxidation. This point is in process of investigation.

SUMMARY

There is a late appearance of carbonic anhydrase in the cerebrum of the young of the dog, the cat, the rat, and the rabbit, animals born in an immature condition; this is in contrast to its presence at birth in the cerebrum of animals more mature when born such as the calf and the guinea pig.

The data suggest correlation between the appearance of the enzyme in the cerebrum and the beginning of its functioning as indicated by the parallel maturation of certain sense organs and the first appearance of α -brain waves, as reported in the literature.

The fact that there is an even later appearance of the enzyme in the central nervous system of the rat, dog, rabbit, and cat than in that of the human infant increases the probability that the finding, previously reported, of no carbonic anhydrase in the cerebrum of the human new-born may be normal.

We wish to acknowledge our indebtedness to Dr. E. C. Rice of the Children's Hospital, Washington, and to the Animal Laboratories of the Naval Medical Research Institute, National Naval Center, Bethesda, Maryland.

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REDUCTION OF α,γ -DIKETO AND α -KETO ACIDS CATALYZED BY MUSCLE PREPARATIONS AND BY CRYSTALLINE LACTIC DEHYDROGENASE

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The enzymatic reduction of a series of normal α,γ -diketo acids by an enzyme present in aqueous extracts of acetone powder from rabbit muscle occurs in a system consisting of diketo acid, enzyme, reduced diphosphopyridine nucleotide (DPNH_2), and phosphate buffer at pH 7.2 (1). Since spectrophotometric determinations reveal that equimolar quantities of DPNH_2 and diketo acid disappear, it has been concluded that only one keto group is reduced. The absence of lactic acid as a product of the reaction eliminates the possibility that hydrolytic cleavage of the diketo acid to pyruvate and fatty acid (2) precedes the oxidation of DPNH_2 . The reduction of α,γ -diketo acids appears to be analogous to the reduction of pyruvic acid to lactic acid. Therefore crystalline lactic dehydrogenase as well as several crude muscle preparations has been studied. In addition to the normal α,γ -diketo acids, several branched chain diketo acids and a number of α -keto acids have been employed in the present investigation.

EXPERIMENTAL

The α,γ -diketo acids and β,δ -diketocaproic acid were prepared and characterized as previously described (2, 3). The normal α -keto acids were synthesized by the procedure of Adickes and Andresen (4). The products were purified by vacuum distillation and gave on analysis the following:¹

α -Ketobutyric acid, $\text{C}_4\text{H}_6\text{O}_3$

Calculated, C 47.05, H 5.93; found, C 47.11, H 6.21

α -Ketovaleric acid, $\text{C}_5\text{H}_8\text{O}_3$

Calculated, C 51.72, H 6.94; found, C 51.35, H 7.14

α -Ketocaproic acid, $\text{C}_6\text{H}_{10}\text{O}_3$

Calculated, C 55.39, H 7.75; found, C 55.46, H 7.94

α -Ketoheptanoic acid, $\text{C}_7\text{H}_{12}\text{O}_3$

Calculated, C 58.30, H 8.39; found, C 58.11, H 8.57

¹ The elemental analyses were performed by Mr. Robert Koegel.

α -Keto-octanoic acid, $C_8H_{14}O_3$

Calculated, C 60.73, H 8.92; found, C 60.40, H 8.74

α -Keto-nonanoic acid, $C_9H_{16}O_3$

Calculated, C 62.77, H 9.36; found, C 62.47, H 9.49

Phenylpyruvic acid was prepared (5) just before use and the experiments carried out within 3 days of its preparation.

Phenylpyruvic acid, $C_9H_8O_3$

Calculated, C 65.83, H 4.91; found, C 65.61, H 4.60

Solutions of the sodium salts of the keto acids were prepared by neutralization with the calculated amount of sodium hydroxide. Pure samples of sodium pyruvate and sodium α -ketoisocaproate were donated by Dr. J. P. Greenstein. Oxalacetic acid was obtained by the procedure of Krampitz and Werkman (6), and ethyl β -ketoundecylate by a procedure analogous to that of Fischer *et al.* (7). The α -ketoglutaric acid was donated by Dr. M. Berenbom, and the *p*-hydroxyphenylpyruvic acid by Dr. G. H. Hogeboom. Ethyl acetoacetate and γ -ketovaleric acid were Eastman products and were distilled several times before use. Solutions of the sodium salts of β -ketoundecylic and acetoacetic acids were prepared as described by Davies (8). Diphosphopyridine nucleotide (DPN), obtained from the Schwarz Laboratories, was purified by counter-current distribution as described by Hogeboom and Barry (9). Despite formation of emulsion, necessitating prolonged centrifugation, a rather pure product was obtained in 75 to 80 per cent yield. The coenzyme, reduced as described by Ohlmeyer (10), was 90 per cent pure. Ohlmeyer's extinction coefficient for $DPNH_2$ was used in all calculations. Triphosphopyridine nucleotide (TPN) of 70 per cent purity and free from DPN was donated by Dr. G. H. Hogeboom and Dr. W. C. Schneider. $TPNH_2$ was obtained by enzymatic reduction of TPN with the theoretical amount of *d*-isocitric acid and a dialyzed extract of an acetone powder of rabbit muscle. The mixture was made 0.1 M with respect to sodium hydroxide, heated at 85° for 5 minutes, cooled, neutralized, and centrifuged before use. The *d*-isocitrate was isolated from *Bryophyllum* leaves (11) generously supplied by Dr. H. B. Vickery.

Acetone powders were prepared as described by Green *et al.* (12) and homogenized with 5 to 10 volumes of cold 0.1 M phosphate buffer at pH 7.2. They were used after centrifugation and dialysis for 2 hours at 5°. Crystalline lactic dehydrogenase was prepared from beef heart as described by Straub (13) and recrystallized four times. Two batches of the enzyme were prepared. Preparation 1 consisted of about 50 mg. obtained from 6 kilos of fresh heart, while Preparation 2 contained about 500 mg. isolated from 50 kilos of heart. The enzymatic data for the two prepara-

tions were almost identical. Most of the data reported below were obtained with the larger preparation. The activity was unchanged after dialysis against 0.04 to 0.1 M phosphate buffer (pH 6.6 to 7.2) for 36 hours at 5°. No loss in activity occurred when the enzyme was stored at 5° for 4 weeks.

Enzymatic activity was determined by following the decrease in the absorption of DPNH_2 at 3400 Å in a Beckman spectrophotometer. The reaction mixture usually contained DPNH_2 , substrate, enzyme solution, and 0.033 M phosphate buffer at pH 7.2 in a volume of 3 cc. in a 1 cm. cell. Suitable dilutions of enzyme or extract were prepared with 0.1 M phosphate buffer at pH 7.2. The reference cell contained all components except DPNH_2 . The reaction was started by addition of 0.05 to 0.2 cc. of enzyme and readings were taken every 15, 30, or 60 seconds. After correction for changes in volume, activity values were obtained from the initial linear portion of the time curve. All experiments were conducted at room temperature (24–27°). In certain experiments with the crystalline enzyme, in which high concentrations of coenzyme were employed, smaller quantities of coenzyme were added to the reference cell in order to bring the reading to a lower point on the density scale. In these experiments the enzyme was omitted from the reference cell. The absorption of the enzyme at 3400 Å is negligible under these conditions. In experiments in which the α, γ -diketo acid concentration was followed, diketo acid rather than DPNH_2 was omitted from the reference cell and readings were made at 2900 Å (2). Since there is a small difference between the absorption of DPNH_2 and DPN at 2900 Å, the values were corrected by subtracting the absorption change due to oxidation of DPNH_2 , which was 10 per cent of the total density change. Although the aliphatic α, γ -diketo acids absorb slightly at 3400 Å, this absorption does not disappear on reduction of the diketo acid, since the density changes at 3400 Å are the same when equimolar quantities of pyruvate and α, γ -diketo acid are added to an excess of DPNH_2 and enzyme. Protein nitrogen was determined by the Kjeldahl technique and protein values were obtained by multiplying the nitrogen values by 6.25.

Results

When the concentrations of α, γ -diketo acid and DPNH_2 were followed simultaneously, equimolar quantities of these reactants disappeared. The data obtained in two typical experiments are given in Fig. 1. When coenzyme was omitted, no disappearance of diketo acid occurred, nor was the coenzyme oxidized in the absence of diketo acid. The reaction did not proceed in the absence of enzyme or with heated enzyme solutions. In confirmation of previous findings (1) no lactic acid was detected (14)

at the conclusion of the experiment. The rate of the reaction with the rabbit muscle preparation increased rapidly with increasing substrate concentration. The optimal substrate concentration for α,γ -diketovaleeric acid under these conditions was $7.5\ \mu\text{M}$ per 3 cc. Because of the large slit width required for measurements with high diketo acid concentrations, a concentration of $5\ \mu\text{M}$ per 3 cc. was usually employed. With limiting amounts of substrate, the curve of coenzyme oxidation against time leveled off at an equimolar quantity of DPNH_2 , indicating that the reaction goes to completion.

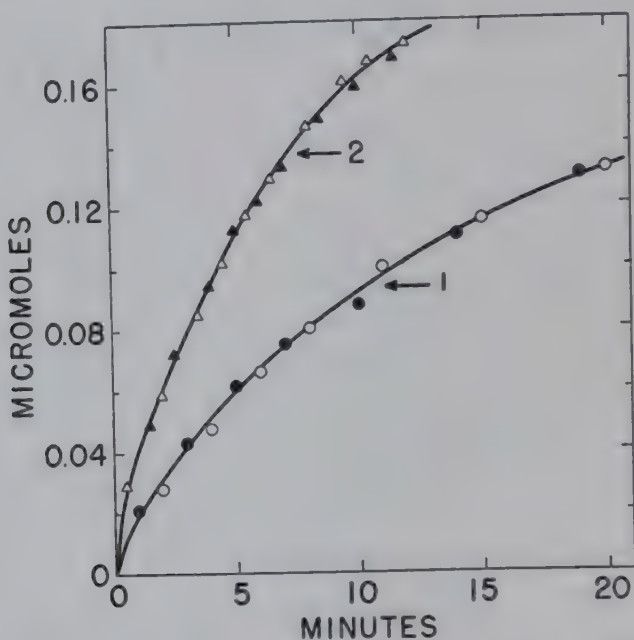


FIG. 1. Relation between disappearance of DPNH_2 and α,γ -diketo acid. Curve 1, α,γ -diketovaleeric acid (initial concentration, $0.158\ \mu\text{M}$ per 3 cc.) with acetone powder extract from rabbit muscle. Curve 2, α,γ -diketocapric acid ($0.222\ \mu\text{M}$ per 3 cc.) with crystalline lactic dehydrogenase. Initial DPNH_2 concentrations were $0.373\ \mu\text{M}$ (1) and $0.425\ \mu\text{M}$ (2) per 3 cc. Solid symbols, DPNH_2 ; open symbols, diketo acid.

The activity of acetone powder extracts of hog heart, pigeon breast muscle, and rabbit skeletal muscle, and of crystalline lactic dehydrogenase toward a number of keto acid substrates was determined. All of the α,γ -diketo and α -keto acids studied were reduced by these preparations (Table I). The relative activity values for the various substrates were about the same for the different enzyme preparations. Of particular interest is the finding that the relative rates with the purified lactic enzyme were essentially the same as those for the crude preparations. The time-activity curves for the α -keto acids were linear for about one-half of their course, while those for the α,γ -diketo acids tended to fall off early after

a relatively short linear period. Although the latter phenomenon has not been investigated fully, it seems possible that inhibition by the product of the reaction, *i.e.* the hydroxyketo acid, may be responsible.

TABLE I

*Reduction of Keto Acids by Muscle Preparations and by Lactic Dehydrogenase**

Keto acid	Rabbit muscle	Pigeon breast muscle	Hog heart	Lactic dehydrogenase	
				Rate	Michaelis-Menten constant, $\times 10^{-5}$ M
α,γ -Diketovaleric.....	28.8	29.4	27.1	3,170 (3400)	41.7
α,γ -Diketocaproic.....	27.2	27.6	25.6	2,880 (3290)	83.5
α,γ -Diketoheptanoic.....	17.6	20.2	14.6	2,180 (2590)	96.0
α,γ -Diketo-octanoic.....	18.4	22.1	17.6	1,980 (2640)	54.4
α,γ -Diketono-nanoic.....	24.8	25.8	23.4	2,780 (3160)	46.0
α,γ -Diketocapric.....	23.2	23.9	19.0	2,760 (3000)	71.1
α,γ -Diketoundecylic.....	19.2	18.4	16.1	2,080 (2400)	50.0
α,γ -Diketo- δ -methylcaproic...				1,040	
α,γ -Diketo- ϵ -methylheptanoic...				1,530	
α,γ -Diketo- δ -dimethylcaproic...				0	
α,γ -Diketo- γ -phenylbutyric...				520	
Pyruvic.....	267	291	300	26,800 (26,800)	5.20
α -Ketobutyric.....	147	184	191	15,300 (21,000)	83.5
α -Ketovaleric.....	22.7	15.3	20.5	1,470 (4840)	334
α -Ketocaproic.....	5.34	5.52	6.15	560 (1400)	300
α -Ketoheptanoic.....	0.934	0.919	1.27	112 (483)	569
α -Keto-octanoic.....	0.466	0.536	0.528	67.0 (160)	167
α -Ketononanoic.....	0.222	0.268	0.293	25.6 (61.5)	167
α -Ketoisocaproic.....				(3.20)	167
Phenylpyruvic.....				615	
<i>p</i> -Hydroxyphenylpyruvic.....				289	
Oxalacetic.....				12.8	
α -Ketoglutaric.....				6.39	

* The rates of reduction are expressed as moles $\times 10^{-8}$ per mg. of protein per minute at pH 7.2 and 26°, at optimal coenzyme concentration. The substrate concentration was 5 μ M per 3 cc. except for α,γ -diketo- γ -phenylbutyrate in which the concentration was 0.625 μ M per 3 cc. The values in parentheses were obtained with optimal substrate concentrations.

The effect of pH on the rate of reduction of several keto acids was investigated with 0.033 M phosphate buffers. With the crude rabbit muscle preparation the reduction of pyruvate proceeded most rapidly at about pH 7.5. On the other hand, maximal activity toward α,γ -diketovaleric acid was observed at about pH 6. The pH-activity curves for these substrates and for α,γ -diketocapric, α -ketobutyric, and α -ketovaleric acids

were determined for lactic dehydrogenase (Fig. 2). The optimal pH for pyruvic and α -ketobutyric acids was about 7.8, while that for the diketo acids and α -ketovaleric acid was approximately 6.

The effect of substrate concentration on the rate of keto acid reduction by lactic dehydrogenase was studied for all of the normal aliphatic α,γ -diketo and α -keto acids. The optimal substrate concentration is lowest for pyruvic acid and greatest for α -ketoheptanoic acid. The variability in substrate-enzyme affinity is reflected in the wide range of the Michaelis-Menten constants listed in Table I. The values for the α,γ -diketo acids were more constant than those for the α -keto acids.

In contrast to the variability of the effects of substrate concentration on rate, the effect of varying DPNH_2 concentration was, over the range

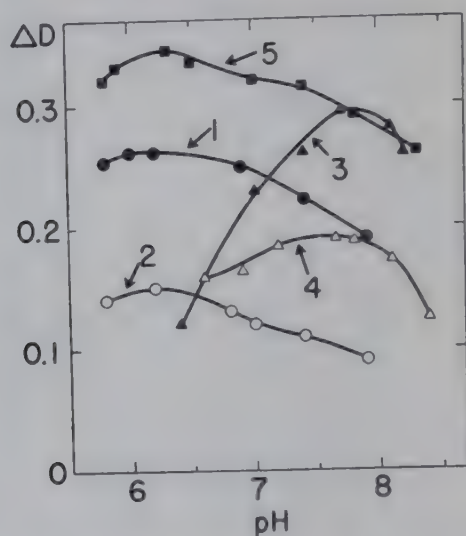


FIG. 2. Influence of pH on reduction of α,γ -diketo and α -keto acids by lactic dehydrogenase. Curve 1, α,γ -diketovaleric acid; Curve 2, α,γ -diketocapric acid; Curve 3, pyruvic acid; Curve 4, α -ketobutyric acid; Curve 5, α -ketovaleric acid at 26° in 0.033 M phosphate buffers. Ordinate, density change per minute.

studied, about the same for pyruvic, α -ketovaleric, α -keto-octanoic, α,γ -diketocapric, and α,γ -diketovaleric acids. The Michaelis-Menten constant was estimated to be less than 10^{-5} M. It seemed of interest to determine whether TPNH_2 could be substituted for DPNH_2 in the diketo acid reducing system. It has been reported that the reduction of pyruvate with crystalline ox heart lactic dehydrogenase proceeds 220 times more rapidly with DPNH_2 than with TPNH_2 (15). It was found that both pyruvic and α,γ -diketovaleric acids were reduced with TPNH_2 . The rates were 100 to 380 times faster with DPNH_2 , depending upon the concentration of coenzyme employed (Fig. 3). The affinity of enzyme for TPNH_2 is much lower than for DPNH_2 . The Michaelis-Menten constant is 4.2×10^{-5} M.

A comparison was made of the rates of reduction by crystalline lactic dehydrogenase of the various keto acids employed with optimal concentrations of substrate and coenzyme. The maximal activity toward the normal α -keto acids falls off rapidly with increasing chain length, although the values for pyruvic and α -ketobutyric acids are fairly close. It was also of interest to compare the rates of reduction of α -keto-*n*-caproic acid with its isomer α -ketoisocaproic acid. A decrease in activity of about 450-fold was observed with the branched chain compound. In contrast to the α -keto acids, the effect of chain length on the reduction rates of α,γ -diketo acids was not as evident. However, lower rates were observed

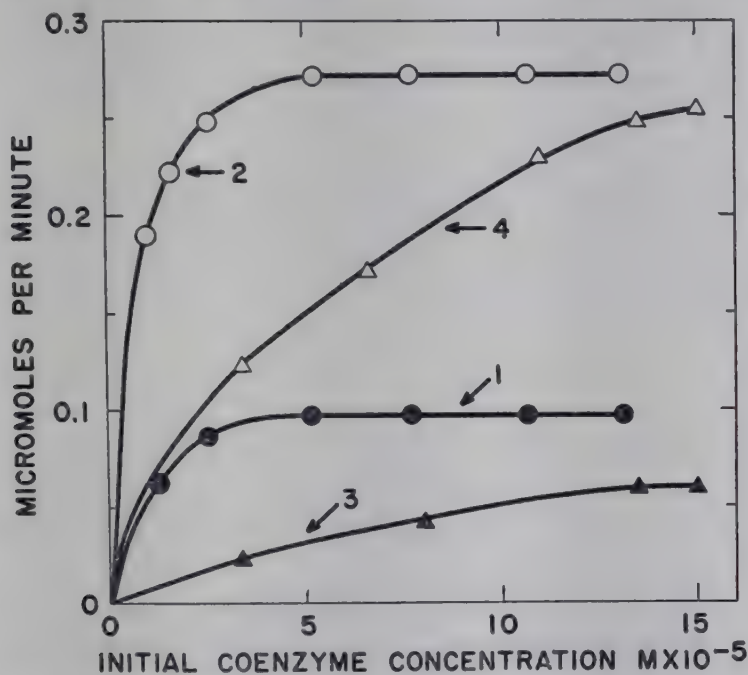


FIG. 3. Effect of DPNH₂ and TPNH₂ concentration on the reduction of pyruvic and α,γ -diketovaleric acids by lactic dehydrogenase. Curve 1, α,γ -diketovaleric acid, DPNH₂, 3.0 γ of enzyme; Curve 2, pyruvic acid, DPNH₂, 1.0 γ of enzyme; Curve 3, α,γ -diketovaleric acid, TPNH₂, 200 γ of enzyme; Curve 4, pyruvic acid, TPNH₂, 150 γ of enzyme. Substrate concentration 5 μ M per 3 cc.; 25°; pH 7.2.

with α,γ -diketo- δ -methylcaproic and α,γ -diketo- ϵ -methylheptanoic acids, while α,γ -diketo- δ -dimethylcaproic acid was completely resistant to reduction.² α -Ketoglutaric and oxalacetic acids were reduced at extremely low rates, and acetoacetic, β -ketoundecylic, γ -ketovaleric, and β,δ -diketocaproic acids were not reduced.²

Several experiments were performed with mixtures of α -keto and α,γ -diketo acids. If the rates of oxidation of DPNH₂ were found to be additive, this result would be compatible with the presence of two separate enzymes. However, in no case was the value obtained for the mixture

² No oxidation of DPNH₂ was observed in 10 minutes with 800 γ of enzyme.

greater than the value for the most rapidly reduced substrate alone. Usually the mixture of α -keto and α,γ -diketo acids oxidized DPNH₂ much more slowly than did the most active substrate alone. A few studies on this inhibitory phenomenon were carried out. In the experiment described in Table II, striking inhibition of pyruvic acid reduction by α,γ -diketovaleric acid occurred. With equimolar concentrations of the two keto acids a value equivalent to about 40 per cent of the value for pyruvic acid alone was observed.

As an approach to the question of the enzymatic homogeneity of crystalline lactic dehydrogenase, ultracentrifugal and electrophoretic studies were carried out.³ Both preparations of lactic dehydrogenase were found to sediment as a single substance (Fig. 4). The sedimentation constants ($s_{20,w}$) were 6.48 and 6.36×10^{-13} Svedberg units for Preparations 1 and

TABLE II
*Inhibition of Lactic Dehydrogenase by α,γ -Diketovaleric Acid**

Pyruvic acid	α,γ -Diketovaleric acid	DPNH ₂ oxidized
μM	μM	moles $\times 10^{-8}$ per min.
0	5.00	1.44
5.00	0	11.7
5.00	0.25	10.5
5.00	0.50	9.03
5.00	1.25	8.45
5.00	2.50	5.19
5.00	5.00	4.22

* The reaction mixtures contained 0.30 μM of DPNH₂, 0.44 γ of lactic dehydrogenase, 100 μM of phosphate buffer at pH 7.2, and keto acids as indicated, in a final volume of 3.00 cc.

2, respectively. The estimated range of molecular weight, based upon these data, is 100,000 to 150,000.

When the enzyme was examined in the Tiselius apparatus, however, two components were observed (Fig. 5). The diagram showed a larger component (A) with a mobility⁴ of -2.64 sq. cm. per volt per second, and a peak (Component B) corresponding to a mobility of -1.92 sq. cm. per volt per second. The slower moving component was estimated to comprise about 15 per cent of the total protein. After electrolysis at 10.37 volts per cm. for 16,680 seconds, samples were withdrawn from the cell with a fine capillary from the positions indicated in Fig. 5. An additional

³ The author is indebted to Dr. Gerson Kegeles and to Mr. Frederick J. Gutter for performing the ultracentrifugal and electrophoretic determinations.

⁴ Determined from descending boundaries.



FIG. 4

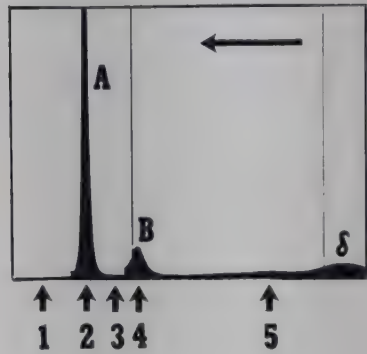


FIG. 5

FIG. 4. Sedimentation diagram of crystalline lactic dehydrogenase (Preparation 1). Protein concentration, 0.9 per cent; phosphate buffer pH 6.60, ionic strength, 0.03; speed 60,000 r.p.m. Photograph taken 47 minutes and 40 seconds after reaching full speed. *M*, meniscus; *B*, bottom of cell.

FIG. 5. Electrophoretic diagram (ascending limb) of crystalline lactic dehydrogenase (Preparation 2). Protein concentration, 1.4 per cent; temperature 4°; phosphate buffer, pH 6.60; ionic strength, 0.1. Photograph taken after electrolysis at 10.37 volts per cm. for 16,680 seconds. The numbered points indicate the positions sampled.

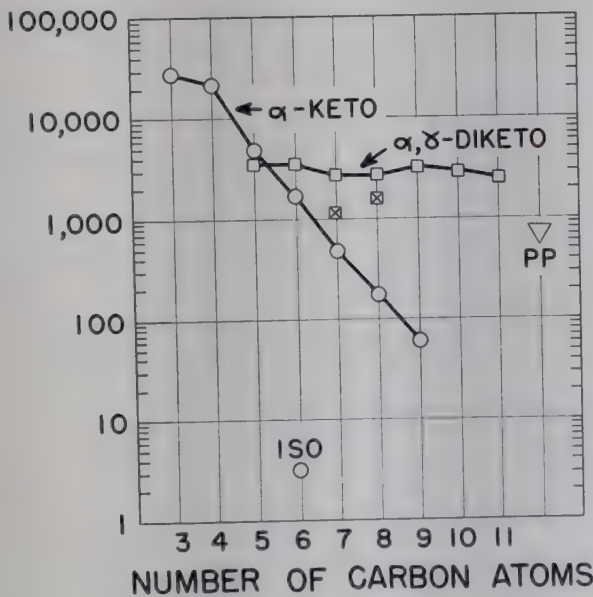


FIG. 6. Relation between carbon chain length and turnover number. Ordinate (logarithmic scale), turnover number (moles of substrate reduced per 100,000 gm. of enzyme at pH 7.2 and 26°). Abscissa, number of carbon atoms in substrate molecule. The curves are drawn for the normal α -keto and α,γ -diketo acids. $\boxtimes$ indicates values for the branched chain α,γ -diketo acids; *ISO* = α -ketoisocaproic acid; *PP* = phenylpyruvic acid.

sample (No. 6) was removed from the horizontal section of the cell. A photograph taken immediately after sampling showed no disturbance of the pattern. The samples were tested against pyruvic, α -ketovaleric, and α,γ -diketovaleric acids. The ratios of activities for the several substrates were, within experimental error, constant in Samples 2 to 6. No activity or protein was found in Sample 1, and the activity to protein values for Sample 6 were very close to those for the original enzyme preparation. The activity to protein values were 19, 17, and 15 per cent greater in Sample 2 than in Sample 6 for α,γ -diketovaleric, α -ketovaleric, and pyruvic acids, respectively. The enzymatic activity is therefore apparently associated with Component A, and the enzyme preparation is estimated to be about 85 per cent pure on the basis of these electrophoretic and enzymatic determinations.

DISCUSSION

Attempts to isolate and identify the product of the reduction of α,γ -diketo acids have been limited by the small quantities of reduced coenzyme available. It has been established, however, that the product of reduction of α,γ -diketovaleric acid forms an alkali-soluble 2,4-dinitrophenylhydrazone which exhibits an absorption maximum at 4250 Å. The product was previously considered to be the corresponding α -hydroxy- γ -keto acid (1). This appears to be the most likely possibility in view of the general susceptibility of α -keto acids to reduction and the failure of γ -ketovaleric acid to be reduced. Fosdick and Rapp (16) reported the isolation of an acid fraction from the fermentation products of pyruvic acid by *Staphylococcus albus*, which yielded a 2,4-dinitrophenylhydrazone possessing a corrected melting point of 218–225° and containing 18.12 per cent nitrogen. When the fraction was treated with hypiodite, a product was found which gave a 2,4-dinitrophenylhydrazone with a melting point close to that of oxalacetic acid. These authors therefore considered the isolated acid to be γ -hydroxy- α -ketovaleric acid. The chemical procedures used for identification by these workers do not appear to eliminate the possibility that the product was α -hydroxy- γ -ketovaleric acid. It seems probable that α -hydroxy- γ -ketovaleric acid would also yield this product by oxidation of the α -hydroxyl group, inasmuch as lactic acid is known to give an iodoform reaction (17).

The evidence suggests that α,γ -diketo and α -keto acids are reduced by the same enzyme. In support of this possibility is the constancy of the activity ratios for the various substrates with preparations of different types of muscle from different species and with crystalline lactic dehydrogenase. Furthermore, the relative rates with various concentrations of DPNH₂ and TPNH₂ for α,γ -diketovaleric and pyruvic acids were approximately the same. The differences in pH optima and Michaelis-Menten

constants are probably a function of substrate structure. It is clear that a pH optimum of about 6 for the reduction of diketo acids is not confined to this class of compounds, for a similar pH-activity curve was observed with α -ketovaleric acid. The ultracentrifugal and electrophoretic evidence also supports the single enzyme hypothesis. However, crystallinity and homogeneity in the electrophoresis apparatus and ultracentrifuge are frequently unreliable criteria of purity (18). The possibility that the two **classes** of keto acids are reduced by separate but closely associated enzymes cannot be discarded. The finding that α -keto acids other than pyruvic are reduced by lactic dehydrogenase is compatible with the observation of Green and Brosteaux that α -hydroxybutyrate was oxidized by a crude dehydrogenase preparation (19). There is also indirect evidence that α -ketoisocaproic and *p*-hydroxyphenylpyruvic acids are reduced by lactic dehydrogenase (12). In view of the wide variety of keto acids reduced by lactic dehydrogenase, its use as a specific method of determination for pyruvic acid (20) would appear to be limited. However, in systems in which the identity of the keto acid is established, the enzyme provides a rapid and convenient method of estimation not only of pyruvic acid but also of other α -keto acids. In the presence of excess enzyme and coenzyme we have been able to determine α -ketobutyric acid and phenylpyruvic acids. Oxalacetic and α -ketoglutaric acids are not likely to interfere with the determination of pyruvic or α -ketobutyric acids, provided too great an excess of enzyme is avoided. The slight activity of the enzyme toward oxalacetic acid may be due to a trace impurity of malic dehydrogenase or to the formation of a small amount of pyruvate from the substrate.

The turnover number for pyruvate for the enzyme used in the present study was 26,800 moles per 100,000 gm. at 26° and pH 7.2. This value is about 31,500 on the basis of the electrophoretic data. Kubowitz and Ott reported a value of 23,000 moles per 100,000 gm. at 20° and pH 7.36 for the lactic dehydrogenases of Jensen's sarcoma and rat muscle (21). They observed Michaelis-Menten constants for pyruvate and coenzyme of 9×10^{-5} M and 5×10^{-6} M, respectively, as compared to 5.2×10^{-5} M for pyruvate and a value estimated to be less than 10^{-5} M for DPNH₂ in the present studies. The ultraviolet absorption curve of the enzyme employed in the present studies is similar to those reported by Kubowitz and Ott with a maximum at 2800 Å.

The effect of the length of the carbon chain on the rate of reduction of α -keto acids is influenced considerably by the presence of a γ -keto group. Although activity falls off rapidly with increasing carbon chain length with the simple α -keto acids, the normal α,γ -diketo acids are reduced at rates of about the same order of magnitude (see Fig. 6 and Table I). The introduction of a single methyl group on the δ - or ϵ -carbon of the α,γ -diketo acid results in a decrease in activity, while complete substitution of

the δ -carbon with methyl groups renders the compound unsusceptible to enzymatic reduction. This is in contrast to the effects of chain modification on the hydrolytic reaction of α,γ -diketo acids (2). Although β,δ -diketocaproic acid is apparently hydrolyzed by the same enzyme that acts upon α,γ -diketo acids (22, 23), only the latter are reduced by DPNH_2 in the presence of crude muscle preparations or lactic dehydrogenase.

SUMMARY

1. The reduction of a series of α,γ -diketo and α -keto acids by aqueous extracts of acetone powders of hog heart, rabbit skeletal muscle, and pigeon breast muscle, and by crystalline beef heart lactic dehydrogenase in the presence of reduced diphosphopyridine nucleotide was studied by a direct spectrophotometric procedure.

2. In the case of the α,γ -diketo acids, one keto group (probably the α -keto group) was reduced to a hydroxyl group.

3. The relative rates of reduction for the various substrates by the several muscle preparations and crystalline enzyme were approximately the same. The rates fell off rapidly with increasing length of the carbon chain for the α -keto acids, pyruvic and α -ketobutyric acids being reduced most rapidly, while the rates for a series of normal α,γ -diketo acids (from valeric to undecylic) were of about the same order of magnitude.

4. The crystalline enzyme did not act upon γ -ketovaleric, β,δ -diketocaproic, or α,γ -diketo- δ -dimethylcaproic acids.

5. The reduction of pyruvic and α,γ -diketovaleric acids proceeded 100 to 380 times more slowly with reduced triphosphopyridine nucleotide.

6. The effects of pH and concentration of substrate, diphosphopyridine nucleotide, and triphosphopyridine nucleotide on the rate of reduction were studied.

7. The reduction of pyruvate by lactic dehydrogenase was markedly inhibited by α,γ -diketovaleric acid.

8. Crystalline lactic dehydrogenase was homogeneous in the ultracentrifuge, but showed two components on electrophoretic examination. The enzymatic activity was associated with the more rapidly moving component, which comprised about 85 per cent of the total protein.

9. Lactic dehydrogenase may be used for the determination of α -ketobutyric, phenylpyruvic, and other α -keto acids in systems in which the identity of the keto acid has been established.

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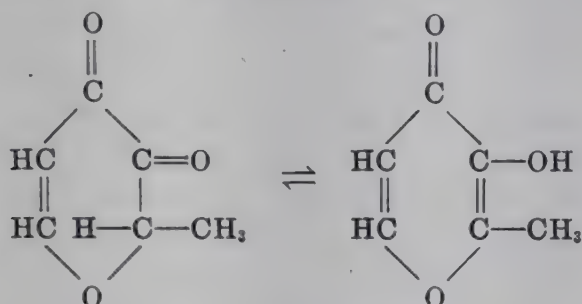
THE FORMATION OF MALTOL* IN CERTAIN CARBOHYDRATE-GLYCINE SYSTEMS†

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(Received for publication, November 7, 1949)

The molecular constitution of maltol, which has been proved by Peratoner and Tamburello (8), is as follows:



This compound occurs naturally in the bark and needles of certain conifers (4, 12). It is also formed during the pyrolysis of such materials as malt, cellulose, starch, and wood (2, 3, 5). It has been stated that maltol is a growth inhibitor (11). Sherman (10) has noted that baked cereals and bread crust give qualitative tests for maltol. More recently, it has been shown that maltol is formed by alkaline degradation of streptomycin (9) and during the prolonged heat treatment of milk (7).

In a previous investigation concerning maltol formation in heated milk, lactose was shown to be the origin of the compound. It was observed further that casein or simply the amino acid, glycine, was capable of converting small quantities of lactose to maltol under the influence of heat, whereas lactose alone would produce no maltol (7).

The fact that maltol could be produced in a lactose-glycine solution suggested that a study of its formation in other carbohydrate-glycine systems might be worth while. Yields of maltol either from natural sources or by pyrolysis of natural products are extremely small. There appears to be no method for the laboratory synthesis of maltol, and further, the mechanism of its formation from carbohydrates is little, if at all, understood. A mechanism for the formation of maltol by alkaline degradation of streptomycin has been proposed by Lemieux and Wolfrom (6).

* 3-Hydroxy-2-methylpyrone-4.

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EXPERIMENTAL

The following carbohydrates were investigated as a source of maltol: starch (refined from corn), cellulose (ashless filter paper), sucrose, maltose, lactose, glucose, galactose, and methyl α -D-glucopyranoside. Carbohydrate-glycine systems, composed of 150 gm. of carbohydrate and 30 gm. of glycine made up to 1 kilo with distilled water, were autoclaved at 127° for 6 hours. Control systems with glycine omitted were also prepared and autoclaved under identical conditions. The pH values of these systems, prior to autoclaving, ranged between 5.5 and 7.2. Those containing glycine had uniform pH values of 7.0 ± 0.2 . Following autoclaving, the samples were cooled, saturated with sodium chloride, and steam-distilled. Maltol, which steam-distills quite readily, gives an intense and stable purple coloration with ferric chloride reagent. This test is sensitive to maltol in concentrations as low as 1 part in 500,000 of water. Therefore, the ferric chloride test was used to detect maltol qualitatively and to follow its steam distillation. The steam distillate was collected, in the instances in which maltol was shown to be present, until the ferric chloride test on the distillate became negative (approximately 5 liters). The distillates containing maltol were extracted with an equal volume of chloroform, a treatment which effectively removed the maltol from the distillate as indicated by the ferric chloride test. The chloroform extracts were concentrated to a volume of 50 ml. by evaporating the solvent, then dried over anhydrous magnesium sulfate, after which the remainder of the chloroform was evaporated. More or less complete crystallization of maltol in the residues was attained by the addition of a small quantity of petroleum ether. The crude yields of maltol, after crystallization for 4 days at -7° , were filtered, washed with petroleum ether, dried, and weighed. The maltol samples thus obtained were evidently fairly pure as indicated by their crystallinity and melting range of $157\text{--}159^\circ$ (Beilstein (1) as 159°). This material was shown to be maltol by melting point on mixture with an authentic sample (from wood tar), donated by the Cliffs Dow Chemical Company, and with a sample of maltol obtained from heated milk, which was characterized by preparation of the benzoate and phenylurethane derivatives.

Of the eight carbohydrate-glycine systems studied, together with their respective control samples, maltol was detected in, and isolated from, only two. 130 mg. of the compound were recovered from the maltose-glycine sample and 55 mg. were obtained from the lactose-glycine sample.

In order to investigate the possibility that maltol might be present in a conjugated form, the methyl α -D-glucopyranoside system was refluxed with 100 ml. of concentrated hydrochloric acid for 2 hours and then steam-distilled again. The lactose-glycine system, after removal of the initial

yield of maltol, was subjected to the same procedure. In neither case did this treatment result in further maltol formation.

In two additional trials with lactose, the glycine was replaced with equal weights of aniline in the first, and oxalic acid in the second. No maltol was detected in either instance.

DISCUSSION

The similarity between lactose and maltose is well known. These two sugars are both disaccharides containing a hemiacetal configuration. The fact that maltol could not be obtained from sucrose or methyl α -D-glucopyranoside also suggests the importance of the hemiacetal structure. Since no maltol was produced in either the galactose or glucose solutions, it would appear that the complete disaccharide molecule is required.

It is interesting to note that maltol and 5-hydroxymethyl-2-furfural have isomeric molecular formulas ($C_6H_6O_3$). Both compounds result from the heating of certain carbohydrates and recently Wolfrom *et al.* (13) have demonstrated the formation of 5-hydroxymethyl-2-furfural in heated glucose-glycine systems. Theoretically, the latter compound results from the removal of 3 molecules of water from the hexose molecule. A somewhat similar mechanism seems indicated for the formation of maltol from lactose or maltose.

Since no maltol was obtained from any of the control samples, it would appear that glycine acts in a catalytic capacity with regard to maltol formation from lactose or maltose in aqueous solution. In the presence of glycine, maltol is formed with much less rigorous heat treatment than that of pyrolysis.

SUMMARY

The formation of maltol upon heating certain aqueous carbohydrate-glycine systems has been investigated. Carbohydrates used in the experiment included starch, cellulose, sucrose, lactose, maltose, glucose, galactose, and methyl α -D-glucopyranoside. Of these, maltol was obtained from only lactose and maltose. Since no maltol could be detected in any of the control samples of carbohydrates, a catalytic rôle seems indicated for glycine in the conversion of the two disaccharides to maltol. Certain implications have been drawn concerning the mechanism of maltol formation in these experiments based on structural similarities and differences between the carbohydrates studied.

The author wishes to thank Dr. A. P. Dunlop of the Quaker Oats Research Laboratories for his interest in this research.

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GRIFOLIN, AN ANTIBIOTIC FROM A BASIDIOMYCETE

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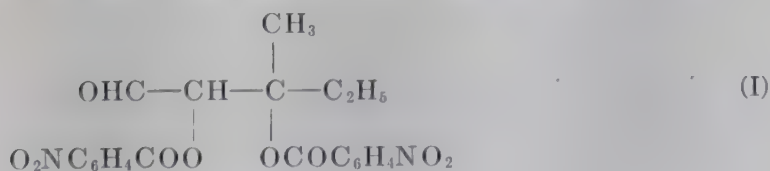
Extensive research concerning the antibiotics obtained from Basidiomycetes is being carried out (1, 2) but, in comparison with products from *Penicillia*, *Aspergilli*, *Actinomyces*, and bacteria, the chemical nature of only a few has as yet been clarified. Furthermore, none has been of practical use, owing to strong toxicity or weak activity.

Kubo and Mizuno (3) have discovered that an antibiotic is produced by *Grifola confluens* (local name, "maitake" or "masugoke") (Fig. 1), which we have extracted in a pure state and have named "grifolin." The present paper deals mainly with its chemical structure.

Grifolin is soluble in most organic solvents but insoluble in water. It is fairly stable towards acid and alkali, but with increasing alkali concentration the solution acquires a purple-red color and resinifies when heated in a 10 per cent alkali solution.

Grifolin, though inactive against Gram-negative bacteria such as *Bacillus anthracis*, *Bacillus dysenteriae*, and *Salmonella typhimurium*, was active against *Staphylococcus aureus* (1:200,000) and *Bacillus subtilis* (1:180,000); the sensitivity of acid-fast bacteria (*Mycobacterium avium*, *Mycobacterium phlei*) to this substance is especially noteworthy. The toxicity is low, the minimum lethal dose for mice being 1.6 gm. per kilo, body weight. The activity is still retained when grifolin is heated to 200°.

Analysis and molecular weight determinations established the empirical formula of grifolin as C₁₆H₂₈O₂. The presence of two hydroxyl groups was demonstrated by esterification with *p*-nitrobenzoyl chloride. Catalytic reduction revealed the presence of three double bonds. Ozonolysis gave the dimer of acetone peroxide, carbon dioxide, acetone, levulinic aldehyde, and acetic acid. Since the fragment containing the hydroxyl groups seemed to be rather unstable, the *p*-nitrobenzoyl ester was likewise submitted to ozonolysis, whereby a water-insoluble product, C₂₀H₁₈O₉N₂, colorless needles from ethanol, m.p. 216°, was obtained. This product appeared to contain two *p*-nitrobenzoyl groups and an aldehyde group. As lead tetraacetate reacts with free grifolin with the formation of methyl-ethyl ketone, it seemed probable that this product had the structure I.



The above results suggest that one end of the structure consists of an isopropylidene group and the other of the glycol corresponding to structure I. In analogy with the structure of natural products, such as methylheptenone and terpenes, the isopropylidene group was thought to be linked with a group

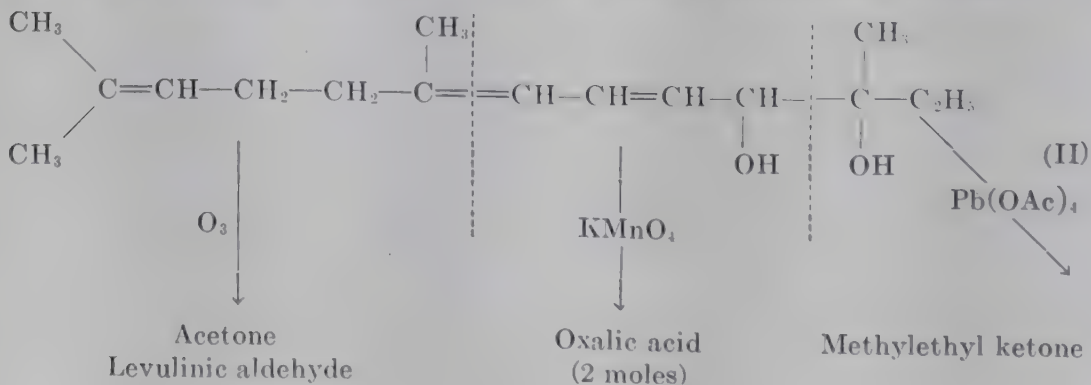


The constitution of the central portion remained to be clarified. This was undertaken by the semiquantitative oxidation with KMnO_4 . Since this reagent, under mild conditions, attacks only double bonds and α -glycols, 2 moles of oxalic acid would theoretically have resulted. Actually the formation of 1.5 moles of oxalic acid and no malonic acid was indicated in



FIG. 1. *Grifola confluens* (photographed by Mr. H. Kubo)

agreement with the proposed structure of grifolin (structure II). This structure explains consistently the qualitative and quantitative results obtained by ozonolysis, KMnO_4 oxidation, and lead tetraacetate oxidation:



The results acquired from the measurement of surface tension of monomolecular films of grifolin are also in accordance with this chain structure.

EXPERIMENTAL

Extraction—The air-dried sporophores were thoroughly dried in a desiccator and ground. 100 gm. of powder were extracted with 95 per cent ethanol until the extract was almost colorless. The solution was concentrated to 35 to 40 cc. and left overnight in an ice box, when white precipitates (mainly mannitol and sterol) separated. These were filtered and 100 cc. of water were added to the filtrate, whereupon a red-brown oily layer and a turbid aqueous layer formed. The aqueous layer was discarded and the washing repeated several times, by which the water-soluble substances and ethanol were removed. The oily layer was dissolved in 100 cc. of methanol and cooled. Some colorless crystals were removed, the methanol was evaporated, and the residue (6 gm. of an oil) shaken with 1 per cent NaOH and ether. The alkali layer was discarded and the washing repeated until the alkali solution remained almost colorless. The ether layer was washed in turn with water, dilute H_2SO_4 , and water, and then dried over CaCl_2 . The red-brown syrup obtained by evaporating the solvent yielded crystals when cooled. For further purification, the dried ethereal solution was directly chromatographed on alumina. The colored impurities were absorbed on the top band and grifolin was obtained from the ether eluate; yield, 2 gm. A slightly modified method, with lead acetate used before the addition of methanol, has been employed in the preparation of larger amounts. In addition to mannitol and sterol, a hemin-like substance (4) and crystals possessing the composition $\text{C}_8\text{H}_{14}\text{O}$ (m.p. 145°) and $\text{C}_{15}\text{H}_{24}\text{O}_2$ (m.p. $151\text{--}152^\circ$) have been obtained as by-products during various extraction procedures.

Purification—Crude grifolin from the eluate was dried under reduced pressure and dissolved in a small amount of petroleum ether (b.p. $40\text{--}60^\circ$). The solution was cooled overnight, rapidly filtered through a previously cooled glass filter, and the product washed several times with ice-cold petroleum ether.

Fine colorless needles, exhibiting a feature characteristic of Liesegang rings, were obtained; m.p. 40° (Fig. 2). Distillation under reduced pressure (150° and 1 mm.) was exceedingly slow and unsuitable as a means of purification.

Analysis— $\text{C}_{16}\text{H}_{28}\text{O}_2$. Calculated. C 76.12, H 11.2
Found. " 75.82, " 9.98
Mol. wt. calculated 252, found 250

*Antibiotic Activity*¹—Grifolin was active against Gram-positive bacteria, against *S. aureus* 1:200,000, and against *B. subtilis* 1:180,000, as measured by the agar streak method. Against Gram-negative bacteria such as *B. anthracis*, *B. dysenteriae*, and *S. typhimurium*, the substance is inactive when the dilution exceeds 10,000. Grifolin inhibits the growth of acid-fast bacteria *in vitro* (Table I). The *p*-nitrobenzoyl ester is inactive.

*Ultraviolet Absorption Spectrum*²—The spectrum, measured with the aid of the Spekker photometer and the iron spark, showed molecular extinc-



FIG. 2. Grifolin ($\times 2$)

tions, $E_{\max.} = 800$ at $275 \text{ m}\mu$ and $E_{\min.} = 375$ at $255 \text{ m}\mu$, with complete transmission above $300 \text{ m}\mu$. When the hydrogen discharge tube was used as the light source, the absorption maximum resolved into two closely adjoining maxima.

p-Nitrobenzoyl Ester—The ester was prepared from crude grifolin with pyridine as the solvent.

¹ The tests were carried out at the Nagoya Industrial Science Research Institute's Laboratory of Antibiotics.

² We are greatly indebted to Dr. K. Yamasaki for the measurements.

The crude ester was dissolved in dry ether and cooled; petroleum ether was added, and colorless needles crystallized. These were recrystallized from petroleum ether; m.p. 62°.

Analysis— $C_{30}H_{34}O_8N_2$. Calculated. C 65.45, H 6.23, N 5.09
 Found. " 66.12, " 6.35, " 5.12
 Mol. wt. calculated 550, found 570

Number of Double Bonds—The method and apparatus used for the determination, with a modified Warburg manometer, were those described by Tsuda and Sakamoto (5). The catalyst was platinum oxide (6), the

TABLE I
Dilution of Grifolin against M. avium and M. phlei

Time of reaction hrs.	<i>M. avium</i> *									
	1000		2000		5000		10,000		50,000	
1	-†	+	+++	+++	+	+	+++	+	+++	+++
3	-	-	-	-	-	-	-	++	+++	+++
5	-	-	-	-	-	-	-	-	+++	+++
9	-	-	-	-	-	-	-	-	-	-
	<i>M. phlei</i> ‡									
	2000		5000		10,000		20,000		50,000	
1										
3	-	+	-	+	+	+	++	++	++	++
5	-	-	-	-	-	-	+	+	+	+++
9	-	-	-	-	-	-	+	+++	+	+++

* Time of incubation, 48 to 52 hours at 37°.

† + growth, - no growth.

‡ Time of incubation, 10 days.

solvent acetic acid. Approximately 5 mg. of grifolin were completely hydrogenated within 30 minutes. The findings indicated 2.49, 3.22, 3.01 (mean, 2.91) double bonds.

Ozonolysis of Grifolin—10 to 15 per cent ozone (washed with alkali and sulfuric acid) was passed for 3 to 5 hours through an ice-cold solution of 5 gm. of grifolin in 50 cc. of chloroform. The solvent was evaporated *in vacuo*, 40 cc. of water were added, and the mixture boiled with a reflux for 1 to 2 hours, while a slow stream of CO₂-free and KMnO₄-washed air was passed through the apparatus into three flasks, the first containing water, and the second and third a saturated Ba(OH)₂ solution. The evolution of CO₂ was soon evident; 0.14 mole of BaCO₃ was obtained. On the addition

of a saturated 2 N solution of 2,4-dinitrophenylhydrazine there was obtained acetone dinitrophenylhydrazone, which, after recrystallization from methanol, melted at 118–120°. The same product was obtained by the ozonolysis of the *p*-nitrobenzoic ester. White crystals (m.p. 132–133°) possessing a pleasant odor and showing positive peroxide tests stuck to the walls of the condenser during this boiling procedure. These properties suggested it to be the dimer of acetone peroxide. This was confirmed by hydrolysis in a sealed tube with 5 N HCl, when acetone was formed.

By distilling one-third of the solution, a second crop of the dimer of acetone peroxide was obtained. Free acetone was also identified in the distillate as its 2,4-dinitrophenylhydrazone. Furthermore, the ether extract of the NaCl-saturated distillate yielded a syrupy mass which crystallized when cooled; m.p. 39°. Its 2,4-dinitrophenylhydrazone (m.p. 154°) was also obtained occasionally. The fractions yielding these unidentified derivatives appear to be by-products.

The remaining hydrolysate was distilled *in vacuo* and 2,4-dinitrophenylhydrazine solution added to the distillate. The precipitate was washed with methanol and recrystallized twice from nitrobenzene; m.p. 232°. The melting point (236°) and analytical data corresponded to the 2,4-dinitrophenylhydrazone of levulinic aldehyde.

Analysis— $C_{17}H_{16}O_8N_8$. Calculated. C 44.35, H 3.48, N 24.35
Found. " 44.66, " 3.63, " 26.82

This was confirmed by the preparation of its semicarbazone from the original hydrolysate and by the production of levulinic acid by the ozonolysis of grifolin ester.

The final residue of distillations was saturated with NaCl and extracted several times with ether. Upon addition of aqueous $NaHCO_3$ to the ether solution, evolution of CO_2 showed it to be acidic. The $NaHCO_3$ solution was acidified with HCl, saturated with NaCl, and again extracted with ether. On evaporation of ether, a brown liquid with an acetic acid-like odor remained. The *p*-bromo- and *p*-phenylphenacyl esters (m.p. 82 and 108° respectively) gave no depression in the melting point when mixed with the corresponding derivatives of acetic acid.

Ozonolysis of p-Nitrobenzoyl Ester—The method and the conditions were the same as those for free grifolin, except that about one-half the time was required. CO_2 , acetone, and acetone peroxide were likewise obtained from the washing flasks and the condenser. A water-insoluble, orange brownish mass described below was removed by filtration.

Two-thirds of the hydrolysate was distilled and a 2,4-dinitrophenylhydrazine solution added to the distillate. The precipitate was separated into methanol-soluble and methanol-insoluble fractions. The former gave

orange-yellow crystals (m.p. 125°) and showed no depression in melting point when mixed with the acetone derivative.

$C_9H_{10}O_4N_4$.	Calculated.	C 45.87,	H 4.23
	Found.	" 44.86,	" 4.95

The latter was the derivative of levulinic aldehyde.

The remaining solution was submitted to distillation *in vacuo*, whereupon a further amount of levulinic aldehyde was obtained in the distillate; acetic acid and levulinic acid (identified as its 2,4-dinitrophenylhydrazone) were obtained from the residue.

The water-insoluble fraction was crystallized first from acetone (an acetone-insoluble fraction is also obtained, which is described below) and then from ethanol; colorless needles, m.p. 216°.

$C_{20}H_{18}O_9N_2$.	Calculated.	C 55.82,	H 4.21,	N 6.51,	mol. wt. 430
	Found.	" 54.15,	" 4.00,	" 6.36,	" " 459

Tests with the fuchsin aldehyde reagent and benzidine suggested the presence of an aldehyde group. The acetone-insoluble fraction (m.p. 166°), dissolved in pyridine, liberated iodine from potassium iodide. Moreover, when dissolved in alkali, it gradually acquired a brownish color with the separation of crystals of *p*-nitrobenzoic acid. Since the same behavior was displayed by the product melting at 216°, it was suspected to be a peroxide of this fragment.

Lead Tetraacetate Oxidation—9 gm. of lead tetraacetate (approximately 5 equivalents) were added to a solution of 1 gm. of grifolin in 20 cc. of anhydrous benzene and left overnight at 37°. The brown precipitate was removed by decantation and the benzene solution was shaken with dilute aqueous $NaHCO_3$. The $NaHCO_3$ solution was shaken with ether, the ether evaporated, and the ether distillate shaken with 2,4-dinitrophenylhydrazine solution. The ether layer was separated and evaporated, when an orange 2,4-dinitrophenylhydrazone remained. This was chromatographed on alumina from benzene. Elution of the main band with benzene containing 1 per cent methanol and evaporation of the solvent yielded orange needles. These were recrystallized twice from methanol; m.p. 118–119°. The residual benzene solution was distilled, and the distillate likewise thoroughly shaken with 2,4-dinitrophenylhydrazine solution. The benzene layer was separated, dried with $CaCl_2$, and chromatographed on alumina. The same orange needles were obtained. These needles showed no depression when mixed with the 2,4-dinitrophenylhydrazone of methyl-ethyl ketone. It had been previously reported (7) that this substance melted at 115°, but our decomposition product as well as the synthetic sample, when purified by chromatography, melted at 118–119°. The fact

that a great excess of lead tetraacetate was required suggested the simultaneous occurrence of other reactions.

KMnO₄ Oxidation; (a) Oxidation, of Ester—Several 2,4-dinitrophenylhydrazones were obtained but the only product identified was acetic acid.

(b) *Semiquantitative Oxidation of Free Grifolin*—504 mg. (2.0 mm) of grifolin were dissolved in KMnO₄-treated acetone and 2.53 gm. (equivalent) of powdered KMnO₄ were added during 1 hour at 0°. The MnO₂ was separated and washed with water until the washings remained clear upon addition of CaCl₂. Acetone was removed *in vacuo* from the acetone solution and the residue added to the aqueous solution. 68 cc. of aqueous solution were obtained.

To 10 cc. of this solution, CaCl₂ was added. The precipitated Ca salt was dissolved in dilute H₂SO₄ and extracted five times with 25 per cent

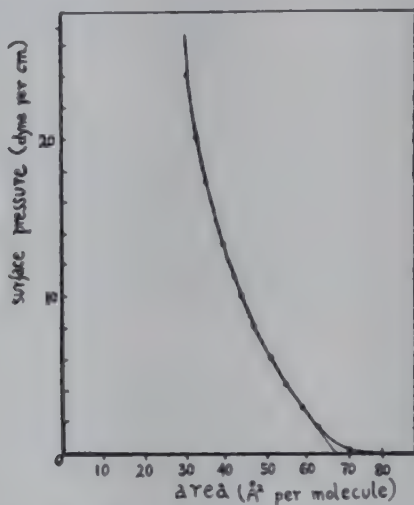


FIG. 3. Surface pressure of the monomolecular film of grifolin (measured at 20°).

ethanol in ether. Upon evaporation of the solvent, white needles remained. They sublimed at 150° and were identified as oxalic acid. Titration with KMnO₄ indicated the presence of 0.31 mm, corresponding to a total of 2.11 mm, of oxalic acid. Titration of the crude calcium salt, without extraction, gave the same results, thus proving that the precipitates were entirely oxalic acid. In a control test with pure oxalic acid, carried out under exactly the same conditions, a 70.5 per cent recovery was obtained. Application of this correction factor leads to a yield value of 1.5 moles of oxalic acid per mole of grifolin.

*Measurement of Surface Pressure*³—A 0.06 per cent benzene solution of grifolin was dropped on distilled water, and the surface pressure of the monomolecular film measured by the vertical plate method (Fig. 3).

³ The authors are greatly indebted to Mr. K. Suzuki for the data obtained.

If the hydrophilic groups in the molecule were assumed to separate, a greater area should have been occupied, since the molecule lies flat on the water surface. Thus it was indicated that the hydrophilic groups occupy adjacent positions. If there were two or more hydrophobic groups extending from the hydrophilic group, the area could not have been compressed below 40 Å, even if it were a substance possessing a narrow *n*-paraffin-like structure. Since the compression was accomplished without accompanying disruption, there could not exist more than two hydrophobic portions. These observations suggest that the molecule does not possess a big ring, and that it has a structure which is capable of being stretched but is not so compact as that of *n*-paraffin with one simple terminal hydrophilic group. The compressibility of the film is therefore consistent with the chemically determined structure of grifolin.

SUMMARY

An antibiotic obtained from the fruit bodies of *Grifola confluens* has been isolated in a crystalline state and named grifolin. Constitutional studies have led to the structure II.

The writers wish to express their appreciation to Professor F. Egami for helpful suggestions throughout this research. They are also indebted to Mr. H. Kubo for continued support and to Mr. S. Nawa and Mr. O. Takeda for technical assistance.

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CONVERSION OF ESTRONE ON INCUBATION IN BLOOD*

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Evidence for the presence in mammalian blood of an estrogen showing phenolic, ketonic, and alcoholic properties was presented in an earlier communication (Werthessen, Baker, and Borci (6)). The biological assay on perfusion experiments indicated that estrogen could be formed from estrone. The estrone used in these studies was dissolved in dilute blood and perfused through an organ (Werthessen (4)).

The degree of estrone destruction was large and in the absence of the organ, *viz.* on incubation of the estrone in blood alone, estrone destruction took place without the formation of the ketol. This unexpected finding was corroborated by polarographic assay. By adapting the polarographic technique to estrogen fractions extracted from blood, we have been able to study aspects of estrone metabolism in this medium with a facility impossible to attain had bioassay been requisite. As the study progressed, it became apparent that a true chemical reaction involving the loss of the 17-ketosteroid nature of the estrone, as well as its biological potency, was under scrutiny. The occurrence of the reaction in a biological medium led to the hypothesis that the reaction was catalyzed by an enzyme. The work to be reported lends considerable support to this hypothesis.

Methods

Extractions of the reaction mixtures followed the scheme devised by Pincus and Pearlman (2) and Pincus and Schiller (3). To decrease the possibility of loss of steroid by oxidation, two modifications have been added: (a) all distillations and evaporations are conducted under nitrogen, and (b) all ether employed in the process is stored over ferrous sulfate until used. In order to prevent possible loss of estrogens by incomplete solution of a tarry residue containing toluene-insoluble ingredients, the alcohol-ether mixture used to extract the estrogens from the perfusate is reduced to a volume of approximately 200 cc. and then toluene is added. Distillation of the three-phase mixture, toluene, alcohol, and water, is continued until the toluene is alcohol-free. Toluene is constantly added during this phase to maintain a volume well over 200 cc.

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In the polarographic determinations, the modified instrument described by Werthessen and Baker (5) has been utilized. The chemical technique described by Wolfe, Hershberg, and Fieser (7) and the more sensitive methods detailed below have been employed.

The following assay composition gave the highest sensitivity and best wave delineation for quantities lying between 50 and 100 γ of estrone per sample (macro-determination): 0.12 cc. of Girard's Reagent T, 100 to 300 mg. per cc., dissolved in anhydrous acetic acid (approximately 30 times the weight of the sample), 1.20 cc. of H_2O , 1.20 cc. of 0.80 N NaOH, and 0.8 cc. of 0.50 N NH_4Cl . For smaller quantities (10 to 50 γ) the following assay composition was employed (micro-determination): 0.03 cc. of Girard's Reagent T, 100 mg. per cc., dissolved in anhydrous acetic acid, 0.30 cc. of H_2O , 0.30 cc. of 0.80 N NaOH, and 0.20 cc. of 0.50 N NH_4Cl .

Girard's reagent dissolved in glacial acetic acid is added to the thoroughly dried residue which previously has been condensed under nitrogen at the base of a conical centrifuge tube. The acid is induced to flow over the entire residue by carefully tilting and rolling the tube. The tube is then heated at 100° for 3 minutes. To the acid is added the water, followed successively by the NaOH and the NH_4Cl solutions. The mixture is thoroughly stirred and then poured into the polarographic assay cell.

In order to obtain reproducible results it was found requisite (a) to use 30 to 40 times as much Girard's reagent by weight as residue assayed, and (b) to make certain that each analysis lay within the limits of the linear relationship between the estrone content of the sample and its electrical conductivity. Use of smaller aliquots of an unknown often tended to show a surprising increment in the total estrone content determined over that in large aliquots. Experience with pure estrone samples demonstrated that if two successively smaller aliquots gave the same total sample value (within 10 per cent) the result could be considered valid.

The factor in the technique requiring the successive smaller samples is not clearly understood at present. Polarographic analysis of organic compounds of the steroid type involves not only the usual polarizing situation, on which the technique was originally based, but also the oxidation or reduction of the material assayed. The system is quite complex but reproducible under properly controlled conditions. The reader is referred to the monograph on polarographic analysis by Kolthoff and Lingane (1) for elucidation of the problems involved.

In view of the need for dilution, it was felt necessary to determine whether a contaminant could influence the actual determinations when the above criteria were observed. Known quantities of estrone were added to various aliquots of residue obtained from a large pooled sample of human blood.

Irrespective of the amount of aliquot employed (and this ranged up to 3 times the maximum blood volume we might use in our regular determination), the error (± 10 per cent) was within that of the technique and instrument.

Estrone was prepared for addition to the perfusate or blood in the following manner: (1) the requisite amount of hormone was dissolved in 95 per cent alcohol, (2) sufficient 0.5 N NaOH was added to the alcohol to render the total volume 20 per cent alcohol, and (3) to this mixture 0.5 N HCl was added to neutralize the NaOH 80 per cent.

The alcohol and NaOH were concentrated enough to sterilize the vessel and its contents. Sterile precautions were observed in the final bottling after addition of the HCl. The solution was stored in rubber-capped sterile vials in the refrigerator. The vials were washed out with sterile blood or blood diluent to insure complete removal of the hormone.

No data reported in Tables I and II have been derived from an experiment which showed any sign of contamination with microorganisms. Their possible presence was checked in each suspicious instance. The ambient temperature of all except a very few experiments was $37^{\circ} \pm 0.2^{\circ}$ or higher. These temperature levels had the advantage in all the incubation experiments of so hastening the rate of bacterial or yeast growth that there was never much doubt as to the presence of microorganisms after 6 or 7 hours or, at the higher temperatures, in 1 hour. The examination of aliquots mentioned above provided the final check on contamination. Any microorganism not capable of extending itself in large numbers throughout as rich a culture medium as blood or blood diluted with White's or Tyrode's solution during 4 to 16 hours of incubation at 37° certainly could not be regarded as capable of affecting the experimental results.

All the fluids used contained penicillin G and streptomycin. These antibiotics in combination were effective in inhibiting the growth of almost all bacteria. They were of course ineffective against the yeasts. The possibility that these antibiotics affect the rate of disappearance of estrone has been checked (1) by the use of blood incubated for a sufficient time to permit destruction of penicillin prior to the addition of the estrone, and (2) by finding that, irrespective of the concentration of antibiotic in the reaction mixture, the result obtained was a function of the concentration of hormone in the blood. This latter point can be seen in the data by noting that the same quantity (50,000 units of penicillin and 50 mg. of streptomycin) of the antibiotics was added to each experiment or to a pooled sample from which blood aliquots were taken to inhibit bacterial growth, whereas the volume of blood and amount of hormone used varied extensively.

Results

Very early in the work the hypothesis that we were dealing with the action of an enzyme system upon estrone became tenable. The study naturally took the course of testing the validity of this hypothesis. The relevant data are presented below.

Demonstration of Presence of Chemical Reaction

In view of the relatively low concentrations of estrone (60 γ per cc. at a maximum) used in these studies and the comparably high concentration of protein usually present, it was requisite to show that simple adsorption on

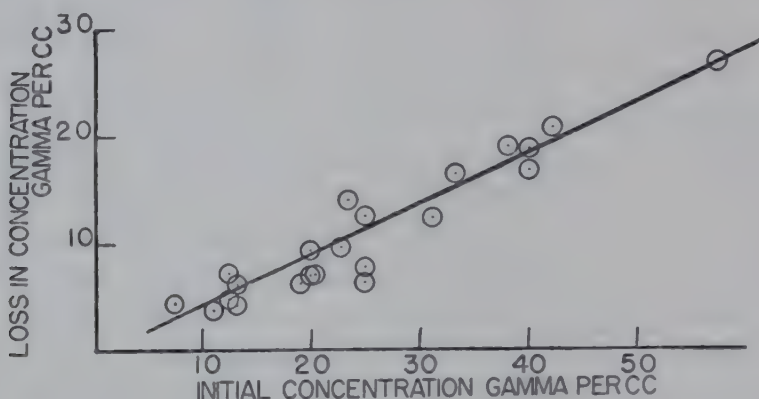


FIG. 1. The relationship between initial concentration of estrone and the loss of estrone concentration expressed as micrograms per cc. The blood used in this study was obtained from normal men and normal premenopause women. It was diluted (50 per cent) with White's solution. The varying concentrations of the hormone were obtained by the addition of constant amounts of estrone (4 or 2 mg.) to varying volumes of the diluted defibrinated blood. 50 mg. of streptomycin and 100,000 units of penicillin were also added to each preparation prior to incubation for 20 to 24 hours. A moistened gas mixture containing 5 per cent CO_2 and 95 per cent O_2 was continuously passed over the system during the incubation. The temperature of the system was maintained at $37.5^\circ \pm 0.1^\circ$.

protein could not account for the findings. The following data considered *in toto* support the hypothesis that adsorption *per se* is an inadequate explanation.

In Fig. 1 are presented data showing the relationship between the initial concentration of estrone and the decrease in that concentration after 16 to 24 hours incubation at $37.5^\circ \pm 0.2^\circ$ under an atmosphere of 95 per cent O_2 and 5 per cent CO_2 . A straight line obviously provides the best fit for the data presented in Fig. 1. This type of relationship is consistent with an equilibrium established in an enzymatic system.

Necessity of Oxygen for Reaction—Since it is most reasonable to assume that oxidation of estrone occurred, the ability of the reaction to proceed

under anaerobic conditions was tested. Three samples of human blood from two donors were shaken under continuously flowing nitrogen for 1 hour prior to the addition of estrone. The fluid was well agitated and equilibration with the nitrogen atmosphere occurred within the hour. The incubation was continued for 16 hours under flowing nitrogen after addition of the hormone. The nitrogen employed contained less than 0.5 per cent O_2 . It was not freed of the remaining trace of oxygen because of the large amounts of gas employed and the concomitant danger that traces of a chemical scrubbing agent could have entered the system during the prolonged incubation. Flowing nitrogen was chosen as the atmosphere because all previous experiments had been run under a continually changing atmosphere. The data are given in Table I.

Three concentrations of estrone (12.8, 24.4, and 48.3 γ per cc.) were employed in the anaerobic incubations (Table I). These concentrations

TABLE I

Loss of Estrone on Incubation in Diluted Blood under Anaerobic Conditions

The blood used was diluted 50 per cent with White's solution. The system was incubated for 16 hours and the temperature was 37.5°. The blood was obtained from two human males; Experiments 294 and 295 employed blood from the same donor.

Experiment No.	Blood volume	Initial concentration per cc.	Loss in concentra- tion per cc.	Expected loss in con- centration per cc.
	cc.	γ	γ	γ
293	78	12.8	7.4	6
294	83	24.1	7.7	11
295	83	48.3	9.7	22

cover the range shown in Fig. 1. The losses found, 7.4, 7.7, and 9.7 γ per cc., are essentially constant, and at the higher concentrations are not inconsistent with losses expected from chemical manipulation alone. Reference to Experiments 421, 431, and 441 in Table II shows that such loss can reach 6.6 γ per cc. at an intermediate estrone concentration (38.8 γ per cc.). Seven experiments to determine the effect of chemical manipulation have been performed. In four of these, 2 mg. of estrone were added to a reasonable volume of alcohol-ether (1800 cc.), and in the remaining three experiments the same quantity of estrone was added to three different volumes of toluene (117, 217, and 317 cc.). The recovery values found ranged from 80 to 85 per cent and averaged 83 per cent. By use of this latter value the expected losses in this experiment should have been 2.2, 4.1, and 8.2 γ . The expected losses in concentration derived from Fig. 1 are 6, 11, and 22 γ , respectively.

These data show therefore that the relation expressed in Fig. 1 between initial estrone concentration and loss in that concentration is dependent on

oxygen for its demonstration (χ^2 for the deviations found, $7.78p = <0.01$). There remains a possibility that the trace of oxygen present in the nitrogen employed accounted for the decrement in concentration observed, especially in the lowest concentration. Elucidation of this point must, in our opinion

TABLE II

Effects of Dilution of Sow Blood with White's Solution on Extent of Loss of Estrone

The total volume of the reaction mixture was 103 cc. 4 mg. of estrone were dissolved in 13 cc. of salt solution as described in the text. The remaining 90 cc. were distributed between White's solution and blood diluted 25 per cent with an anti-coagulant. The initial estrone concentration was 38.8 γ per cc.

Experiment No.	Blood volume	White's solution, volume	Length of incubation	Amount recovered	Estrone loss per cc.
In air					
	cc.	cc.	min.	mg.	γ
412	80	10	30	2.32	16.2
413	50	40	30	2.32	16.2
414	30	60	30	2.65	13.1
415	20	70	30	2.88	10.8
416	10	80	30	2.66	13.0
417	5	85	30	2.66	13.0
418	1	90	30	3.20	7.7
420	0.2	90	30	3.20	7.7
421	0.0	90	30	3.99	0.01
In 5% CO ₂ -95% O ₂					
422	80	10	480	1.82	21.1
423	50	40	480	1.66	22.7
424	30	60	480	1.99	19.5
425	20	70	480	1.33	25.9
427	5	85	480	1.66	22.7
429	0.5	90	480	1.66	22.7
430	0.2	90	480	1.75	21.8
431	0.0	90	480	3.32	6.6
432	80	10	30	2.32	16.2
433	50	40	30	2.32	16.2
440	0.1	90	30	2.30	16.4
441	0.0	90	30	3.34	6.4

await technical refinements in the assay procedure. As is seen from inspection of Fig. 1, the variation in recovery in the lower concentrations is considerable.

Extreme Dilution—Discussed in detail below is a series of dilution experiments. The results of one of these are brought into the discussion at this

point to emphasize the inadequacy of adsorption on protein as an explanation of the loss of estrone.

In one of the dilution experiments a total of 1.5 mg. of alcohol-precipitable solids in blood was allowed to act on 4.0 mg. of estrone. A loss of 1.6 mg. of estrone was found.

The data presented in Fig. 1, the need of oxygen, and the fact that 1.5 mg. of total blood solids can effect the conversion of 1.6 out of 4 mg. of estrone are best explained by assuming that a true chemical reaction is involved.

Effects of Dilution

The capacity to catalyze a reaction when present in minute concentrations has always been considered characteristic of an enzyme system. This characteristic has been tested in the following experiments.

Blood from two sows was used. The experiments were divided into three groups. One group was run under air as atmosphere for 30 minutes, another for the same incubation time under 95 per cent oxygen and 5 per cent carbon dioxide, while the third group was incubated for 480 minutes under the latter gas mixture. Two important points are evident from the data presented in Table II. The first is that over an 8 hour period a blood concentration as low as 0.20 cc. per 100 cc. (Experiment 430) produced as much estrone loss as a concentration of 80 cc. per 100 cc. (Experiment 422). The second point is that the reaction is well under way at the end of $\frac{1}{2}$ hour (Experiments 412, 413 to 417, and 432 to 440).

The rapidity with which the reaction occurs provides an explanation for the parallel recoveries found when 80 cc. and 0.2 cc. of blood acted on estrone for an 8 hour period. Reference to Fig. 1 shows that on even further incubation (16 hours) and approximately 50 per cent blood concentration an expected loss of 18 γ per cc. occurs at the estrone concentrations used in these experiments. This value is in close agreement with the values (22.3 γ average) found in the 480 minute experiments described in the preceding paragraph. The difference (4.3 γ) is certainly within the experimental error.

The inference can therefore be drawn that even with as low a dilution as 0.2 cc. of blood there is sufficient enzyme present to permit the reaction to reach its equilibrium point within 8 hours. Half an hour, however, provided insufficient time to permit approach to the equilibrium point. This is shown in part, though not significantly, by Experiments 432, 433, and 440 which demonstrate a loss of 16.3 γ on the average as compared to the 22.3 γ mentioned above for the 480 minute experiments.

Experiments 412 to 420, with blood from another sow, illustrate the effect of dilution in more marked fashion. Here as the dilution of the blood in-

creased from 80 in 103 parts to 0.2 in 103 a continual decrement in loss is observed. In fact the loss observed changed from what can be considered real (13.0 γ per cc.) to what is explicable by manipulation (7.7 γ per cc.) when 5 and 1 cc. of blood were employed. This result is in complete agreement with that expected when an enzyme concentration is reduced below a critical point under experimental conditions providing insufficient time for small concentrations to demonstrate activity.

Since each of these three series of dilutions contained as a control one flask in which estrone was added to the diluent alone and in which no comparable loss occurred (Experiments 421, 431, and 441), the degree of loss of estrone cannot be ascribed to the diluent, streptomycin, penicillin, or the extraction procedure.

As a final check on the possibility that adsorption played a significant rôle in the observed phenomena, the following dilution experiment was performed. Step 1, the total amount of alcohol-precipitable solids in a sample of sow blood diluted with White's solution was determined. Step 2, another aliquot of the diluted blood was further diluted with White's solution until only 1.5 mg. of the previously determined solids were present per cc. Step 3, to 1 cc. of this dilution were added 89 cc. of White's solution and 4 mg. of estrone in 13 cc. of the alcoholic saline solution described above, bringing the total concentration of blood solids to about 0.015 mg. per cc. and the estrone concentration to about 0.04 mg. per cc. Step 4, a comparable sample was set up so that 150 mg. of solid were present in the same volume. To this 4 mg. of the estrone described in Step 3 were added. Step 5, both samples were incubated together under 5 per cent CO_2 and 95 per cent O_2 for 23 hours at 37.5°.

2.4 mg. of estrone were recovered from the flask containing 1.5 mg. of precipitable material, and 1.2 mg. from that containing 150 mg. These results are comparable to those in the second group in Table II (Experiments 422 to 441) as the same blood was used throughout. The 1.2 mg. recovery in the latter group from the flask containing 150 mg. of precipitable material is in good agreement with the values (1.3 to 2.0 mg.) found in the 8 hour run (Experiments 422 to 430).

Contrariwise, the 2.4 mg. recovered after 23 hours incubation from the sample containing 1.5 mg. of total solids is consistent with the 2.3 mg. recovered with 80 per cent blood in the 30 minute experiments (Table I, Experiments 432 to 440). This is especially notable when it is realized that the 80 per cent blood contained approximately 400 mg. of protein making it therefore 270 times as concentrated as in the run with 1.5 mg. The amount of estrone disappearing (1.7 mg.) is significantly higher than the 0.01 to 0.69 mg. lost in control runs with no blood (Table II, Experiments 421, 431, and 441).

The results obtained in these dilution experiments are therefore in agreement with the hypothesis that they are due to a chemical reaction catalyzed by an enzyme system. They also suggest that the recovery values obtained on prolonged incubation are due to completion of a reaction whose equilibrium point occurs at a level at which approximately 45 per cent of the substrate remains unaltered.

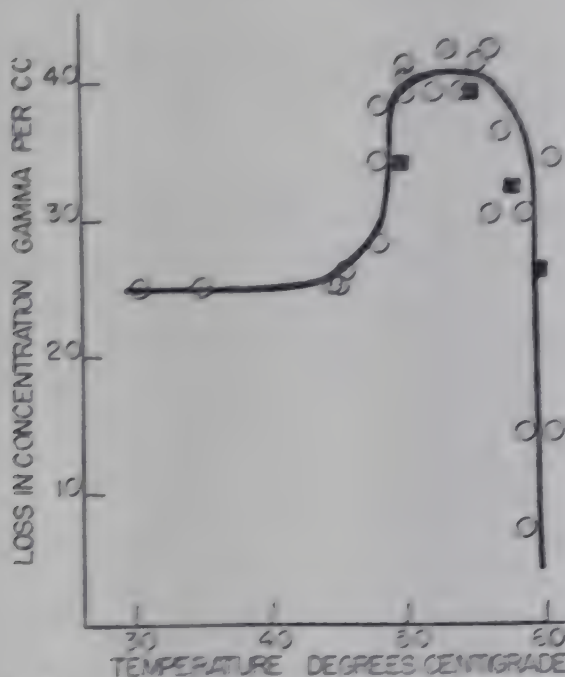


FIG. 2. The effect of temperature on the conversion of estrone by blood. Blood taken from three cows, O, and one human female, ■, was used in this study. It was diluted 50 per cent with White's solution and defibrinated. Temperature equilibration lasted 1 hour prior to hormone addition. The hormone was incubated for $\frac{1}{2}$ hr. 50 cc. of diluted defibrinated blood, 13 cc. of alcoholic saline containing 4 g. of estrone, and 10 cc. of the diluent used to wash out the vials containing estrone comprised the reaction system. Air was used as the atmosphere.

Temperature Sensitivity

The effect of temperature on the reaction has been studied. The data are presented in Fig. 2. Blood taken from three cows and one human male was used in this experiment. The results are presented in a form comparable to that of Fig. 1; *i.e.*, the loss of concentration in micrograms per cc. has been determined and the values are plotted against the temperature of the run. Exactly 50 cc. of blood were placed in a sterile Erlenmeyer flask under air as an atmosphere. The flask and its contents were put into constant temperature bath held at the desired temperature. Equilibration to temperature lasted exactly 1 hour. The estrone (4.0 mg.) was then

added and the two solutions thoroughly mixed. Precisely $\frac{1}{2}$ hour after addition of the estrone, the total volume (73 cc.) was poured into 18 volumes of alcohol-ether.

The curve shown in Fig. 2 is that to be expected from a system containing a heat-labile protein as a constituent of the reaction. The rate of reaction (*viz.* micrograms lost per cc. in $\frac{1}{2}$ hour) increases rapidly from 37–50°. It remains relatively constant to 55° and then falls rapidly as 60° is approached.

Of particular interest is the fact that the four points obtained with blood from a human female fall on the curve as well as can be expected.

SUMMARY

In view of (1) a definite relationship between the concentration of substrate (estrone) and the loss of substrate, (2) the necessity for the presence of oxygen for the reaction to proceed, (3) the occurrence of the reaction at a relatively unchanged rate when the protein content of the system was markedly reduced, and (4) obtaining of a temperature response curve typical for enzymes of a protein nature, it appears reasonable to postulate the presence in blood of an enzyme capable of inducing the oxidation of estrone to a form whose nature is unknown at present.

The data available do not indicate the specificity of the factor nor the nature of the end-products of the reaction. The data of this paper do show that the 17-ketosteroid group of estrone is destroyed. As previously shown (6), this is accompanied by a loss of biological activity.

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BIOAUTOGRAPHIC STUDIES WITH USE OF LEUCONOSTOC CITROVORUM 8081*

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It has been demonstrated by Sauberlich and Baumann (1) that *Leuconostoc citrovorum* 8081 requires an unknown growth factor in its nutrition. The factor was found in parenteral liver extracts, yeast, and in other natural materials. Thymidine was found capable of partially fulfilling the need for the unknown factor.

That the unknown factor is not vitamin B₁₂ was indicated by Lyman and Prescott (2, 3) who showed that the growth factor active for *Lactobacillus leichmannii* (vitamin B₁₂) could be separated electrolytically from the *L. citrovorum* factor. Using paper chromatograms, Sauberlich (4) reported the separation of thymidine from the *L. citrovorum* factor.

It was of interest to examine various preparations reported to contain the *L. citrovorum* factor by the bioautographic technique. With this method it has been possible to show that the factor of Sauberlich and Baumann is multiple in character.

EXPERIMENTAL

The bioautographic technique comprises the separation of various alternative growth factors on paper chromatograms and the recognition of their positions on such chromatograms by use of a microbiological indicator. The value of the method in the recognition of alternative growth factors present in complex materials has been noted previously by the present authors (5, 6).

In this study it has been found that wet symmetrical collidine is a useful solvent for separating the various members of the complex of growth factors utilized alternatively by *L. citrovorum*. *n*-Butanol, a solvent employed by Sauberlich (4), hardly moves the *L. citrovorum* factors from the site of application of the test sample.

While this manuscript was in preparation, a paper by Broquist, Stokstad, Hoffmann, Belt, and Jukes (7) appeared in which these authors made use of the bioautographic method in studies on *L. citrovorum* 8081. They used butanol-acetic acid as the solvent for chromatographic development. By the use of collidine we have been able to show the com-

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plexity of the group of factors active for *L. citrovorum*, in contrast with the results of the above authors. In our hands preliminary studies indicate that even with butanol-acetic acid (prepared according to Partridge (8)) one can demonstrate the multiple character of the *L. citrovorum* complex of growth factors in crude materials. Since most of our experience has been obtained with collidine, results with this solvent are detailed in this paper.

10 to 30 μ l. samples of solutions of various materials to be described were chromatographed on Whatman No. 1 or No. 4 paper strips for 18 or 42 hours by use of the descending technique. The strips were air-dried and then laid on nutrient agar seeded with *L. citrovorum* 8081. The nutrient agar had the same composition as that described (9) for *L. leichmannii* 313 except that folic acid was left out of the medium.

The organism was carried in broth similar in composition to that used in the agar plates, with an amount of Reticulogen added to give maximum growth. An 18 hour culture was centrifuged and washed twice with sterile saline. An entire washed culture from 10 ml. of broth was used to seed 400 ml. of melted nutrient agar at 45°.

After allowing the paper strip chromatograms to rest in contact with the surface of the agar for 5 minutes, they were removed and the agar plate was incubated overnight at 37°.

In a test of this kind 10 μ l. of a folic acid solution containing 100 γ per ml. produced no zone of growth of *L. citrovorum* 8081, in the 18 hour incubation period.

Sauberlich and Baumann (1) reported that folic acid (at a high concentration) would replace the unknown factor in the nutrition of *L. citrovorum*, but only after a long lag period, no growth having been observed in the first 24 to 36 hours.

Our results and those of Sauberlich and Baumann thus indicated that the presence of folic acid in natural extracts would not confuse the picture in studies on the complex of growth factors active for *L. citrovorum*.

Results

In Fig. 1 are shown exact diagrams of a series of bioautographs of samples of Reticulogen of different concentrations. The rate of movement was highest in the case of the two most concentrated solutions. This fact is of some technical importance, since in studies on the *L. citrovorum* complex of growth factors one should not rely simply on the rate of movement of a factor as a means of identifying it. A more unequivocal method involves chromatography of an unknown sample with and without an added reference material. Reticulogen itself has been used in these studies as the standard of reference, principally because it was also used by

Sauberlich and Baumann. Reticulogen apparently contains one major growth factor. However, evidence for the presence of a second slow moving factor is seen in the bioautograph of the most concentrated sample.

In Fig. 2 is shown a series of bioautographs of a 10 per cent solution of Wilson's liver powder 1:20. Strip 1 in Fig. 2 indicates the presence of three *L. citrovorum* factors in this preparation.

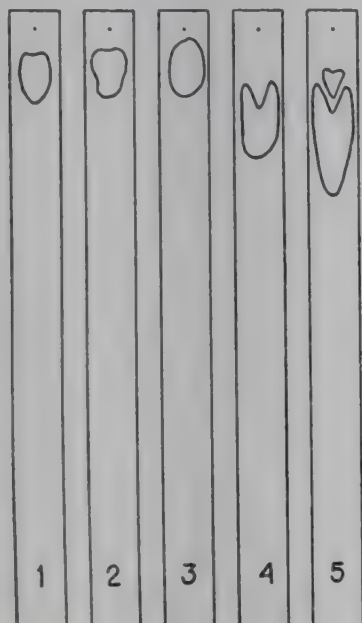


FIG. 1



FIG. 2

FIG. 1. Bioautographs of different dilutions of Reticulogen (Lilly). 10 μ l. samples were chromatographed on Whatman No. 1 paper for 18 hours with wet 2,4,6-collidine as the mobile phase. Test organism, *L. citrovorum* 8081. Strip 1, 1:20 dilution; Strip 2, 1:10 dilution; Strip 3, 1:5 dilution; Strip 4, undiluted; Strip 5, double strength.

FIG. 2. Bioautographs of Wilson's liver powder 1:20 with and without added Reticulogen. Samples chromatographed on Whatman No. 4 paper for 18 hours with wet 2,4,6-collidine as the mobile phase. Test organism, *L. citrovorum* 8081. Strip 1, 20 μ l. of a 10 per cent solution of Wilson's 1:20 liver powder; Strip 2, double strength Reticulogen; Strip 3, combination of Wilson's liver powder 1:20 and Reticulogen, each in same respective concentrations as for Strips 1 and 2.

The two slowest moving factors are normally poorly separated in short term chromatographic experiments. The third and most rapidly moving factor is evidently the same as the main factor in Reticulogen. The latter substance also contains a minor growth factor (minor as to concentration), as is evident in Fig. 2, Strip 2 (see also Fig. 1). All four factors are seen in Strip 3 of Fig. 2, a bioautograph of a mixture of Wilson's liver powder extract and Reticulogen.

In Fig. 3 is a series of bioautographs of a yeast extract and a second parenteral liver preparation. Yeast extract is seen to contain principally

a slowly moving factor (or factors) and a small amount of the factor found as the main constituent in Reticulogen. It is of interest that the factor in the yeast extract which moves moderately rapidly is not seen in the bioautograph of the combination of yeast extract and Reticulogen. Undoubtedly it is present within the large Reticulogen factor zone (in



FIG. 3

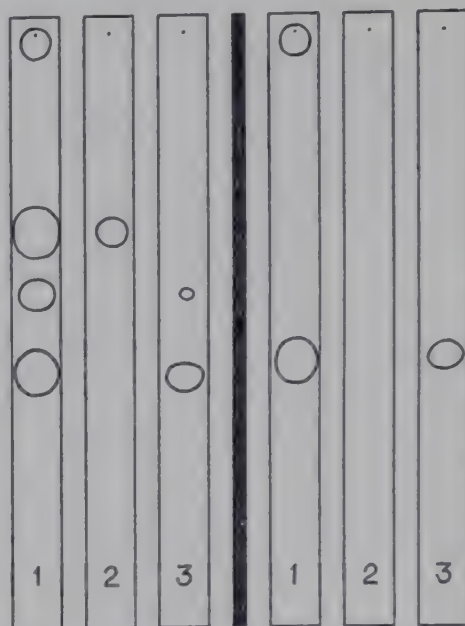


FIG. 4

FIG. 3. Bioautographs obtained with *L. citrovorum* 8081 as test organism. Period of development, 18 hours on Whatman No. 4 paper with wet 2,4,6-collidine as the mobile phase. Strip 1, yeast extract (Difco), 20 μ l. of a 10 per cent solution; Strip 2, yeast extract plus Reticulogen; Strip 3, 30 μ l. of a parenteral liver preparation (Armour); Strip 4, same as Strip 3 with added Reticulogen.

FIG. 4. Comparison of bioautographs obtained with *L. leichmannii* 313 with those obtained with *L. citrovorum* 8081. Chromatograms were developed on Whatman No. 1 paper with *n*-butanol as the mobile phase. Similarly numbered strips represent bioautographs of the same material. Strips 1, 20 μ l. of a parenteral liver preparation (Lederle); Strips 2, 20 μ l. of a solution containing 100 γ per ml. of hypoxanthine desoxyriboside; Strips 3, 20 μ l. of a solution containing 20 γ per ml. of thymidine. The bioautographs on the left were obtained with *L. leichmannii* 313 as the test organism; those on the right with *L. citrovorum* 8081.

Strip 2), with which it is probably identical. The effect of concentration on the rate of movement of this factor has already been pointed out.

The bioautographs of the second parenteral liver preparation (Fig. 3, Strip 4) show the presence of a main growth factor which is probably not the same as the principal factor in Reticulogen.

In Fig. 4 a series of bioautographs obtained with *L. citrovorum* 8081 is compared with those obtained for *L. leichmannii* 313 (9). Butanol was the developing solvent. Thymidine is active for both organisms, con-

firming the findings of Sauberlich and Baumann. Alternative desoxyribosides (including hypoxanthine desoxyriboside) found in a parenteral liver extract were inactive for *L. citrovorum* 8081 but promoted the growth of *L. leichmannii* 313. Kitay, McNutt, and Snell (10) have previously reported that hypoxanthine desoxyriboside is inactive for *L. citrovorum*.

It has been suggested by Sauberlich and Baumann (1) that the *L. citrovorum* factor (or factors) may be related to folic acid, and Sauberlich (4) has shown that feeding rats folic acid resulted in an increased excretion of the *L. citrovorum* factor:

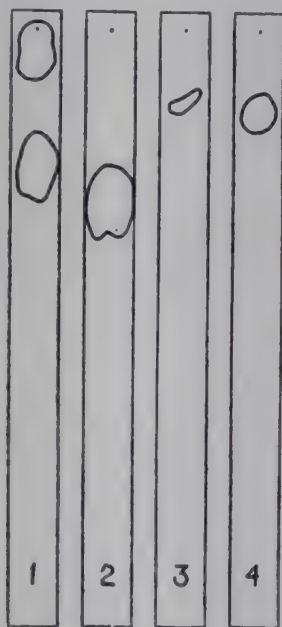


FIG. 5. Bioautographs showing the effects of incubation of various substances with rat stomach homogenate. Samples chromatographed on Whatman No. 4 paper for 18 hours with wet 2,4,6-collidine as the mobile phase. Test organism, *L. citrovorum* 8081. Strip 1, 20 μ l. of a 5 per cent solution of Wilson's liver powder 1:20; Strip 2, 20 μ l. of a 5 per cent solution of Wilson's live; powder 1:20, after incubation with rat stomach homogenate; Strip 3, 20 μ l. of rat stomach homogenate diluted to concentration existing in sample of Strips 2 and 4; Strip 4, 20 μ l. of a sample containing folic acid, 50 γ per ml., after incubation with rat stomach homogenate.

In order to throw light on the site of formation of the *L. citrovorum* factor *in vivo*, an experiment was carried out in which folic acid was incubated with rat stomach homogenate under toluene for 72 hours at 37°. The stomachs of four normal, mature, albino rats from our breeding colony were homogenized in a Waring blender with 10 ml. of cold, distilled water. To 1 ml. of the mucosa homogenate, 1 ml. of folic acid solution (100 γ per ml.) was added. As a control, 1 ml. of homogenate was diluted with an equal volume of water. Both samples were incubated as described above.

Bioautographic analysis of the folic acid reaction mixture indicated the

formation of the *L. citrovorum* factor in excess of that present in rat stomach homogenate alone. This is evident from Strips 3 and 4 in Fig. 5.

The effect noted has not been obtained consistently with rat stomach homogenates prepared at different times, owing possibly to inactivation of the necessary enzyme system during preparation of the homogenates.

However, wherever the bioautographic test has shown the increased size of the zone of growth, this has been confirmed by quantitative assay for the total activity according to methods suggested by Sauberlich and Baumann (1). In a typical experiment, approximately twice as much activity was found in the rat stomach-folic acid mixture (equivalent to 33 μ l. of standard Reticulogen per ml.) as in the rat stomach homogenate itself (equivalent to 16.5 μ l. of standard Reticulogen per ml.). Whether the increased activity found is due to a direct action of the enzyme converting folic acid to the *L. citrovorum* factor cannot be stated.

Evidence of the presence of an enzyme in rat stomach homogenate, which appears to convert chromatographically slowly moving factors to a more rapidly moving factor is also shown in Fig. 5. To 1 ml. of a 10 per cent solution of Wilson's liver powder 1:20, 1 ml. of rat stomach homogenate, prepared as already described, was added. The mixture was incubated at 37° under toluene for 72 hours. As a control, a 5 per cent solution of the liver powder was also incubated under toluene. Strip 1 in Fig. 5 represents the bioautograph of the liver solution incubated in the absence of rat stomach homogenate. It is to be noted that the chromatographic process had not separated the two substances found at the top of the chromatogram in Fig. 2. Strip 2 in Fig. 5 shows the effect of incubation with rat stomach homogenate on the types of factors found in the liver preparation. It is apparent that the slowly moving factors present in the control sample of Strip 1 are absent in Strip 2. (Only a trace zone of growth was visible on the agar plate in the region normally occupied by the slow factors.) In a separate experiment, incubation of a solution of the liver preparation with heated rat stomach produced no effect on the chromatographic picture found normally (as in Strip 1). It is suggested that the disappearance of the slowly moving factors upon incubation with rat stomach homogenate is due to their enzymatic conversion to the fast moving factor found in Strip 2.

Quantitative assay of a solution of Wilson's liver powder before and after incubation with rat stomach homogenate indicated that such incubation caused a doubling of the total *L. citrovorum* activity measured in terms of Reticulogen. Thus a 5 per cent solution contained the equivalent of 119 μ l. of Reticulogen per ml. before incubation; after incubation with rat stomach it contained the equivalent of 236 μ l. of Reticulogen per ml. While part of the increased activity might have stemmed from the action

of the first enzyme, discussed earlier, on the folic acid in liver powder, it is doubtful that the entire increase in activity in the present experiment can be ascribed to such action. It is suggested that the chromatographically slow factors in liver powder may have less activity on a molar basis than the faster factor present alone after incubation with rat stomach homogenate.

Conceivably the chromatographically slower factors represent conjugated forms of the more rapidly moving factor, to which they are converted by an enzyme in the rat stomach homogenate.

These results have a bearing on any tests of the *L. citrovorum* complex of growth factors for possible antipernicious anemia or other physiological activity. In such tests it will be important to know with which members of the complex one is dealing.

SUMMARY

1. A bioautographic method suitable for ascertaining the presence of alternative factors supporting the growth of *L. citrovorum* 8081 has been described. Evidence has been presented for the existence of four such factors, in addition to thymidine.

2. It has been demonstrated that incubation of folic acid with rat stomach homogenate leads to an increase in the amount of one of the factors utilized by *L. citrovorum*. This has been shown by bioautographic analyses and by quantitative tube assays.

3. Evidence for the presence of an enzyme in rat stomach homogenate, which causes the disappearance of the chromatographically slower factors of liver with a concomitant increase in *L. citrovorum* growth-promoting activity, has been offered. After incubation only a chromatographically faster factor was found. It has been suggested that the chromatographically slower factors represent conjugated forms of the faster factor.

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DIETARY FACTORS RELATED TO LIVER XANTHINE OXIDASE*

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In a previous study (1) it was shown that dogs maintained on a purified diet developed a defect in acetaldehyde metabolism. Of the five known enzyme systems that could theoretically be involved in acetaldehyde metabolism *in vivo* (aldolase, mutase, carboxylase, aldehyde oxidase, and xanthine oxidase), the xanthine and aldehyde oxidases seemed most likely to be dependent upon a dietary factor not already incorporated in the purified rations used. Our attention was therefore directed to a study of the relationship between liver xanthine oxidase in rats and the dietary factors supplied to the animals. In this way it was observed that some unidentified factor had to be supplied in the diet in order to obtain normal levels of xanthine oxidase in the liver (2).

Previous studies have implicated both riboflavin (3) and protein (4) as essential dietary factors required by the rat for the establishment of normal xanthine oxidase levels in the liver. Inanition has also been shown to decrease liver xanthine oxidase in the rat (5). Keith *et al.* (6) reported that the incorporation of less than 1 mg. per cent of folic acid in a purified diet decreased the xanthine oxidase activity of chick livers, but as far as rat liver xanthine oxidase is concerned, the feeding of folic acid or 6-pteridylaldehyde is without effect.

EXPERIMENTAL

Xanthine oxidase activity was determined in rat livers by the method of Axelrod and Elvehjem (3). A study of this method was carried out simultaneously with the dietary experiments reported herein, and the results showed that the activity measurement reflected the true order of magnitude of the xanthine oxidase content of the liver (7). All manometric determinations were run in duplicate and the final values for xanthine

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oxidase activity were expressed in units (c.mm. of O_2 consumed per gm. of dry liver per hour).

21 day-old rats were obtained as needed from the Albino Farms and Sprague-Dawley, Inc.; all others were from our own stock, originally Albino Farms animals. Both males and females were used without any detectable difference in response, but males were used predominantly. Early in this study a few 21 day-old animals from each group were sacrificed immediately for analysis of liver xanthine oxidase. The remainder was placed on one of the diets to be described for varying periods of time before their livers were similarly analyzed. Groups of six rats were generally used initially for each point studied, although larger groups were

TABLE I
Purified Diet For Xanthine Oxidase Studies

	gm.
Casein (vitamin test)*	21
Crisco	4
Wesson oil	2
Cod liver oil	1
Salts (Phillips and Hart†)	4
Glucose	68
	mg.
Choline chloride	100
Nicotinic acid	2.5
Calcium pantothenate	1.0
Riboflavin	0.4
Thiamine	0.4
Pyridoxine	0.4

* Obtained from General Biochemicals, Inc.

† Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).

required in many instances to establish a difference as statistically significant.

All of the purified diets used were based upon the 21 per cent purified casein diet shown in Table I; all such diets were identical except for the specific differences noted. The 8 per cent casein diet contained 81 per cent glucose. The peanut protein diet contained 8 per cent casein, 22 per cent defatted peanut meal (contributing 13 per cent peanut protein and 1.5 per cent fat), 0.5 per cent Wesson oil, and 60.5 per cent glucose. The albumin diet contained 21 per cent dried egg albumin, instead of the casein, plus 0.15 mg. per cent of biotin, 0.5 per cent Na_2HPO_4 , and 67.5 per cent glucose. 10 per cent Wilson's whole dried liver was added to the 21 per cent purified casein diet at the expense of glucose. Dried whole milk powder and whole fluid milk were supplemented with a daily intake

of 42 γ of Mn and Cu, and 420 γ of iron as appropriate salts. Purina dog chow, containing 21 per cent protein, was also fed as a natural unpurified ration.

All the purified 21 per cent protein diets gave similar growth curves of about 3.0 gm. per day. Purina dog chow and the 10 per cent liver diets gave 3.0 to 4.0 gm. growth gains per day, while an increase of approximately 2.5 gm. per day was obtained with the milk diets. Growth on the 8 per cent casein diet averaged 0 to 0.5 gm. per day in weanling rats, while feeding the 8 per cent casein diet to adult rats resulted in appreciable weight gains due to an obvious deposition of fat.

Results

The livers from twelve normal adult rats maintained on Purina dog chow had an average xanthine oxidase activity of 1553 units with a standard error of the mean of ± 68 units. At birth, rat livers were devoid of xanthine oxidase. No activity was found in seven pooled samples, each of which contained all the livers from an entire litter of six to ten rats. At the same time, five of the livers of mother rats had an average xanthine oxidase activity of 1247 ± 101 units. At 12 days of age small amounts of activity were found (0 to 300 units), indicating that the necessary dietary factors were supplied in the mother's milk. At 21 days of age, the starting point for the dietary experiments, the liver xanthine oxidase activity averaged 717 ± 116 units.

The results of feeding various diets to 21 day-old rats are shown in Fig. 1. The natural rations gave nearly normal levels of xanthine oxidase within 2 weeks. Purina dog chow, fluid milk, whole milk powder, and the 10 per cent whole liver diet gave values of 1535 ± 97 , 1385 ± 38 , 1535 ± 140 , and 1292 ± 72 units, respectively. Feeding a 21 per cent crude casein diet resulted in a significant increase in liver xanthine oxidase to 1260 ± 65 units after 4 weeks, but not within 2 weeks. When the purified 21 per cent protein diets containing casein, casein plus peanut protein, or albumin were fed, the starting level of liver xanthine oxidase remained essentially unchanged (500 to 1000 units) for at least 6 weeks. Feeding the purified diet for 7 weeks, followed by dog chow for 3 weeks, gave 1433 ± 44 units. The fluctuations observed on the three purified diets during the first 6 weeks were not significantly different from the values obtained with weanling rats or from each other. The 40 to 66 per cent higher values obtained with dog chow, fluid milk, milk powder, and 10 per cent liver within 2 weeks were all significantly higher than the 921 ± 77 units obtained with the 21 per cent purified casein diet in 2 weeks ($P = < 0.01$).

Increasing the protein content of the purified diet to a total of 25 per

cent and 30 per cent did not give appreciably different results from those obtained with the 21 per cent protein diets. These diets contained 8 per cent casein and the remaining 17 per cent and 22 per cent protein were contributed by defatted peanut meal at the expense of glucose. The livers of weanling rats placed on these diets were analyzed in groups of six after 2, 4, and 6 weeks. No differences were found in the groups with increasing dietary periods, and all of the rats on the 25 per cent protein diet averaged 1031 ± 65 units of liver xanthine oxidase. The 30 per cent protein diet gave 1108 ± 67 units as compared with the value of

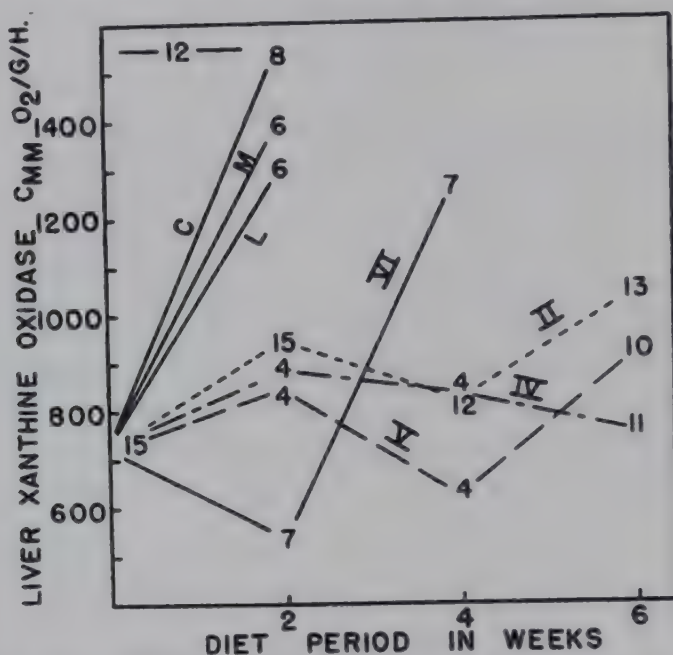


FIG. 1. Liver xanthine oxidase activity after feeding weanling rats the indicated diets for 2 to 6 weeks. The figures at each experimental point refer to the number of rat livers averaged to obtain the point. Curve C, Purina dog chow; Curve M, milk; Curve L, 10 per cent liver; Curve II, 21 per cent purified casein; Curve IV, 8 per cent casein + 13 per cent peanut protein; Curve V, 21 per cent egg albumin; and Curve VI, 21 per cent crude casein.

927 ± 40 for all rats on the 21 per cent casein diet during the first 6 weeks ($P = 0.2$ and 0.03). The results obtained with liver and milk could not be attributed to the higher protein content of these diets. Supplementing the 21 per cent purified casein diet with 0.5 per cent methionine or 1 per cent cystine insignificantly increased the control level by 4 per cent ($P = 0.8$) and 15 per cent ($P = 0.3$), respectively.

Additional results of feeding experiments are shown in Fig. 2. An 8 per cent casein diet depleted the liver of xanthine oxidase, irrespective of the initial xanthine oxidase activity (Fig. 2). It also depleted the xanthine oxidase activity of adult rat livers, but at a somewhat slower rate (8).

Rats depleted of liver xanthine oxidase by a low protein diet restored this enzyme when the dietary essentials were supplied. Xanthine oxidase activities of 0 to 100 units obtained with a low protein diet were restored to 1361 ± 92 units by feeding dog chow for 4 weeks (Fig. 2). The 21 per cent purified protein diets gave values of 1000 to 1100 units under similar circumstances, which represented a remarkably good but incomplete restoration of the liver xanthine oxidase.

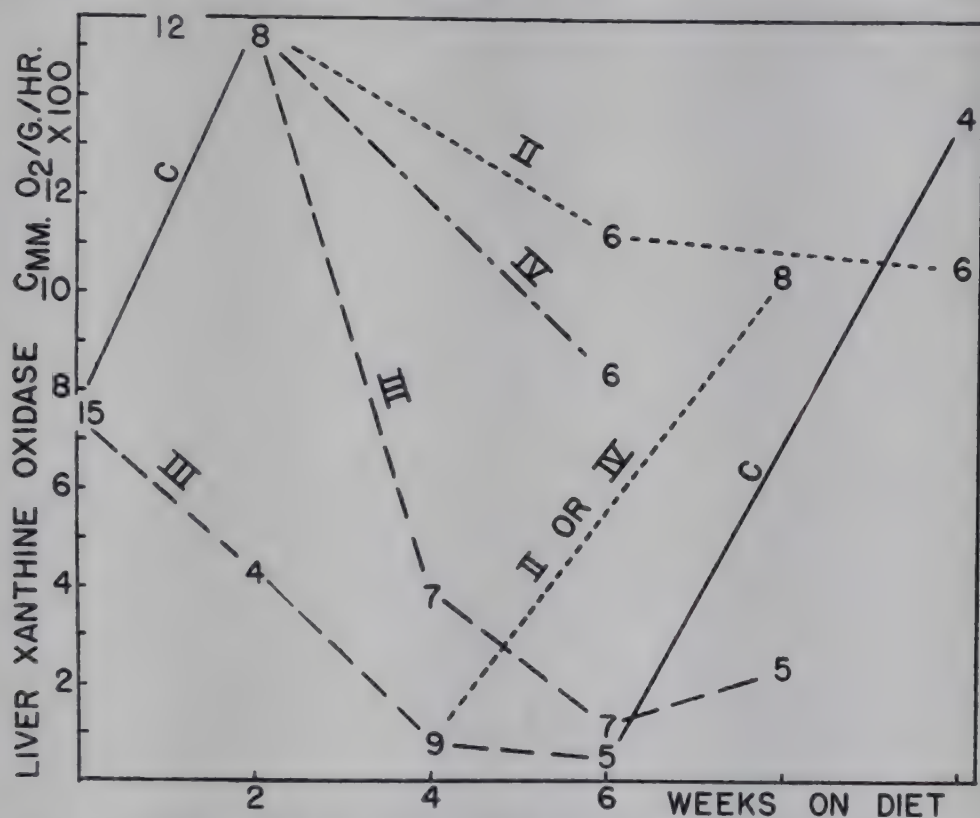


FIG. 2. Changes in liver xanthine oxidase activity that resulted from alterations in the diet. Weanling rats were fed Purina dog chow (Curve C) or Diet III for 2 and 4 to 6 weeks respectively; the diets were then changed as indicated. The figures at each experimental point refer to the number of rat livers averaged to obtain the point. Curve II, 21 per cent purified casein; Curve III, 8 per cent casein; and Curve IV, 8 per cent casein and 13 per cent peanut-protein.

Weanling rats brought to a normal level of xanthine oxidase activity by feeding dog chow for 2 weeks could be partially depleted of this enzyme by feeding the 21 per cent purified protein diets. The casein diet reduced the xanthine oxidase activity to 1055 ± 81 units in 8 weeks, while the peanut-protein diet resulted in livers with an average activity of 832 ± 84 units in 4 weeks. These results were significant decreases from the dog chow level ($P = <0.01$).

Diets containing sucrose or starch instead of glucose were fed to three

groups of six weanling rats each for 2 weeks. The liver xanthine oxidase activities were 613 ± 98 , 771 ± 94 , and 822 ± 88 units respectively for the sucrose, glucose, and starch diets ($P = 0.3$ and 0.7 in comparison with glucose). Adding liver to the sucrose diet gave normal xanthine oxidase levels in 4 weeks (the earliest tested; Table II). Hence the nature of the carbohydrate in the diet did not appear to be of major importance in this relationship.

The fat content of the diet was increased 2- and 3-fold by proportional increases in the three lipid constituents. The fat was added at the expense of glucose, and in other tests the casein and salts were simultaneously increased so that the intake of these constituents per 100 calories of diet remained the same as in the original control diet. When fed for 2 weeks to weanling rats, none of the higher fat diets gave liver xanthine oxidase levels that were significantly different from the simultaneous control group. The values for the 21 per cent fat diet with and without adjustment of the casein and salts intake were 1025 ± 119 and 939 ± 116 units, respectively, in comparison with the simultaneous control values of 814 ± 88 ($P = 0.2$ and 0.4).

Test Supplements—The 21 per cent purified casein diet was supplemented with known dietary factors and fed to groups of six weanling rats for 2 weeks without obtaining significant increases in liver xanthine oxidase above the control level established by the purified diet alone. The supplements tested and the percentage increases (plus per cent) or decreases (minus per cent) from the control values were as follows: (a) biotin 0.25, inositol 10.0, *p*-aminobenzoic acid 10.0, rutin 2.0, ergostanyl acetate 2.0, and folic acid 1.0 mg. per cent gave +4 per cent ($P = 0.8$); (b) biotin 0.25, inositol 10.0, and *p*-aminobenzoic acid 10 mg. per cent gave -25 per cent ($P = 0.2$); (c) riboflavin 1.2, adenine 10.0, *D*-ribose 20.0 mg. per cent gave -14 per cent ($P = 0.3$); (d) I 1.0, Zn 10, Mn 20, Cu 10, and Co 5 mg. per cent as appropriate salts gave -27 per cent ($P = 0.3$); (e) Armour's pernicious anemia extract (≈ 100 gm. of fresh liver per 100 gm. of diet) gave -12 per cent ($P = 0.5$); (f) Armour's vitamin B₁₂ concentrate (≈ 100 per cent fresh liver) administered orally and subcutaneously gave +14 per cent ($P = 0.06$) and +7 per cent ($P = 0.6$) respectively; and (g) 5.0 mg. per cent of folic acid gave -3 per cent ($P = 0.8$). The injection of 0.175 γ daily of Merck's crystalline vitamin B₁₂ for 4 weeks while feeding the purified diet gave +13 per cent ($P = 0.3$).

Xanthopterin gave erratic results which could not be reproduced consistently. Xanthopterin was tested in twenty groups of six rats each, by adding it to the diet in amounts varying from 0.05 to 1.0 mg. per cent or injecting it subcutaneously in amounts varying from 0.002 to 0.1 mg. daily for total dietary periods ranging from 10 to 28 days. Of the twenty

tests, four were considered to be positive or highly suggestive responses, but a repetition of these tests, together with all the others, was clearly negative. One of the positive or highly suggestive tests was obtained by feeding 0.1 mg. per cent of xanthopterin for 19 days, followed by 9 days of control diet alone, before the livers were analyzed and found to contain 1301 ± 148 units of xanthine oxidase; the simultaneous control group averaged 866 ± 77 units. The probability of this being chance variation was about 1 in 40. While xanthopterin was the only pure compound tested that yielded positive or suggestive results, the erratic nature of the response and the preponderant weight of negative evidence suggest some indirect action of xanthopterin or an incomplete supplementation as the explanation for the few positive tests observed.

The following compounds related to xanthopterin were fed at a level of 0.2 mg. per cent in the diet for 2 weeks with negative results: 6-methylisoxanthopterin, α -dihydroxanthopterin, β -dihydroxanthopterin, isoxanthopterin, 7-methylxanthopterin, and 2-amino-4-hydroxypteridine; a simultaneous group fed 0.2 mg. per cent of xanthopterin was also negative.

Compounds Related to Folic Acid—The effect of adding folic acid and 6-pteridylaldehyde to the diet was studied. *In vitro*, the latter compound was a potent inhibitor of xanthine oxidase (9, 10), and the incorporation of folic acid in a purified diet has been reported (6) to decrease the liver xanthine oxidase activity in chicks. Both compounds were added to the purified 21 per cent casein diet and to the diets that otherwise gave normal xanthine oxidase levels in the liver; *i.e.*, dog chow, 10 per cent liver, and whole milk powder. Any increase or decrease in liver xanthine oxidase could thereby be detected. In additional tests, the liver was wetted and autoclaved for 1 hour at 15 pounds before it was mixed with the other dietary components in order to destroy any enzymes that might possibly alter these pteridyl compounds.

The diets were fed to weanling male rats for 4 weeks; the results are shown in Table II. The following points may be noted. The liver xanthine oxidase activity of the rats on the purified diet fell between 800 and 1000 units in the presence or absence of folic acid and 6-pteridylaldehyde. Adding 1 per cent sulfasuxidine to the purified diet gave the same results as the purified diet alone. All of the groups of rats on Purina dog chow, liver, or milk averaged above 1300 units, and in no case did the addition of these pteridyl compounds significantly increase or decrease this activity. Heating the liver did not elicit a response from the pteridyl derivatives; nor did it destroy the factor in liver responsible for increasing the xanthine oxidase activity above the levels obtained with a purified diet. Feeding the rats Purina Startena for 4 weeks gave 1539 ± 119 units, a normal level of activity in rats, in contrast with the very low xanthine

oxidase activity observed in chicks on this diet (6). Obviously the xanthine oxidase in rat liver did not respond to these particular diets in the manner reported for chick livers.

Additional Observations—In confirmation of the work of Miller (5), significant decreases in liver xanthine oxidase were produced by fasting. Fasting for 6 days gave 1115 ± 60 units in comparison with 1540 ± 110 units observed simultaneously in a group of fed rats. A 50 per cent decrease appeared to be about the maximum effect of prolonged fasting, as observed in near fatal fasts of 12 days. In comparison, the 8 per cent

TABLE II

Liver Xanthine Oxidase Activity in Rats Maintained for 4 Weeks Post-Weaning, on Diets Indicated

The numbers in parentheses indicate the number of animals used.

Diet	Xanthine oxidase activity: mean $\pm$ s.e.		
	Unsupplemented diet	Diet supplemented with	
		1 mg. per cent folic acid	0.25 mg. per cent 6-pteridylaldehyde
	units	units	units
Purina dog chow.....	(6) 1639 ± 129	(6) 1414 ± 46	(7) 1616 ± 87
21% purified casein*.....	(12) 897 ± 90	(6) 998 ± 146	(6) 809 ± 200
10% liver*.....	(6) 1343 ± 159	(6) 1597 ± 146	
10% heated liver*.....	(10) 1903 ± 195	(6) 1529 ± 55	(6) 1627 ± 35
Whole milk powder†.....	(4) 1728 ± 260	(5) 2053 ± 220	(6) 1484 ± 136

* Diet as given in Table I, except that sucrose was used instead of glucose. 10 per cent whole dried liver replaced an equal amount of sucrose. The heated liver was wetted and autoclaved for 1 hour at 15 pounds before mixing with the other constituents.

† Dried whole milk powder (Klim, Borden Company) supplemented with 8 mg. of pyridoxine, 8 mg. of thiamine, 30 mg. of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 470 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 38 mg. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per 2.26 kilos.

purified protein diet fed *ad libitum* removed practically all of the enzyme from the liver.

In general, livers with a low xanthine oxidase activity were pale in color, while the very active livers were much darker. Even adult rats that were changed from a chow diet to the purified 8 per cent casein diet lost some color from the liver concomitantly with the decreased xanthine oxidase activity. This correlation of color with activity may be significant or it may be chance association, since all of the diets giving little color and low xanthine oxidase activity to the liver were purified, while the dark, active livers were obtained on rations containing natural foodstuffs.

Over 1000 rat livers have been analyzed for xanthine oxidase activity in

connection with these and related experiments, and some idea of the consistency of the results has been obtained. Individual rats under identical dietary conditions show considerable variation in liver xanthine oxidase, with any one group of six identically treated rats often showing a spread of 1000 units from the lowest to the highest value. In spite of such wide individual fluctuations, the averages of such groups are reasonably reproducible. For example, many groups of six control rats fed the 21 per cent purified casein diet for 2 weeks have been analyzed; none of these averages ever exceeded 1100 units, and most of them fell between 800 and 1000 units. The average for the first fifteen rats analyzed was 921 ± 77 , and an additional eighteen rats gave little change, the over-all average for the thirty-three rats being 877 ± 50 . Similarly, many groups of rats maintained on Purina dog chow have all given averages above 1300 units, usually between 1400 and 1800 units. There can be no doubt that a real difference exists between the effects of these two diets (11), but it is obvious that no importance can be attached to the relatively minor fluctuations observed from group to group on the same diet or to small differences between groups on different diets. We have accepted differences between groups as significant only when they were run simultaneously and when a statistical analysis of the results showed the probability of chance variation to be less than 1 in 50.

Occasional groups of animals maintained on the purified 21 per cent casein or stock chow diets gave values for liver xanthine oxidase appreciably different from the usual ranges. For example, several groups of weanling rats fed the purified diet for 2 weeks had unusually low averages of 300 to 400 units instead of the 800 to 1000 units commonly obtained. Occasional groups of rats maintained on dog chow had activity values over 2000 units instead of the 1400 to 1800 units usually observed. Low values occurred more frequently during the summer months. The reasons for such wide fluctuations among similarly treated animals are not yet clear, but the results obtained with such atypical groups have generally been discounted. The need for simultaneous controls in any study of liver xanthine oxidase is evident, not only because of animal variation but also because of the change in the activity of the xanthine solution itself with aging (7).

Sprague-Dawley Rats—The results previously cited were obtained with rats from the Albino Farms. The limited data available indicate that Sprague-Dawley rats responded similarly, but with some quantitative differences. The average xanthine oxidase activity of eighteen rats at 21 days of age was 468 ± 30 units. Feeding dog chow for 2 and 4 weeks gave 1030 and 1356 units respectively. Feeding the 21 per cent purified casein diet for 4 weeks resulted in an activity of 1123 units. After 3 weeks

on the 8 per cent casein diet, the activity was 26 units; such values were restored to 1133 units after 2 weeks on dog chow, as compared with 479 units on the purified 21 per cent casein diet. In general it appeared that Sprague-Dawley rats required a longer dietary period to reach normal levels of liver xanthine oxidase and that the differences between the purified and natural foodstuffs may not have been as great as when the Albino Farms rats were used.

DISCUSSION

Preparations of xanthine oxidase from milk (12) and liver (13) contain an unknown substance in the prosthetic group in addition to flavin-adenine dinucleotide. The evidence for this can be summarized as follows: (1) The color of the enzyme is golden brown instead of the bright yellow that is typical of flavoproteins; (2) approximately one-third of the absorption at 450 $m\mu$ is due to flavin, the remainder being due to the unknown component; (3) addition of hypoxanthine bleaches the flavin portion of the prosthetic group, but does not remove all of the color; (4) the activity of the apoenzyme, prepared by prolonged dialysis of the enzyme, can be restored by a coenzyme extracted from xanthine oxidase but not by flavin-adenine dinucleotide or flavin phosphate. The evidence presented in this paper shows that some unidentified factor must be supplied in the diet in order for the weanling rat to deposit and retain normal levels of xanthine oxidase in the liver. No evidence has yet been obtained that the required dietary factor is related to the unidentified component of xanthine oxidase, but the possibility is obvious.

Ball (14) has pointed out that more and more flavoproteins with atypical absorption spectra and presumably containing two or more prosthetic groups are being discovered. The list would include two yeast flavoproteins (14, 15), xanthine oxidase (12, 13), liver aldehyde oxidase (16), glucose oxidase from *Penicillium notatum* (17, 18), and quinine oxidase (19). In no case has the non-flavin portion of the prosthetic group been identified, but Ball (14) has indicated that in one of the yeast flavoproteins its absorption spectrum is reminiscent of the pteridine compounds.

SUMMARY

Rats are born without any detectable xanthine oxidase activity in the liver, even when the mother's liver contains normal amounts. About half of the normal adult level was present at weaning.

Feeding weanling rats a purified 21 per cent protein diet for 6 weeks gave little or no increase in the liver xanthine oxidase. Increasing the protein or fat content of the diet or changing the type of carbohydrate fed was similarly without effect. Incorporating 10 per cent whole liver

in the diet or feeding natural rations such as Purina dog chow and milk gave nearly normal levels of liver xanthine oxidase within 2 weeks. Supplementing the purified diet with the known crystalline vitamins was without effect, while xanthopterin gave erratic results.

A purified 8 per cent casein diet depleted the liver of xanthine oxidase when fed to rapidly growing or adult rats. The liver enzyme could be restored to normal by feeding the necessary dietary factors. Rapidly growing rats were also partially depleted of liver xanthine oxidase by feeding a purified 21 per cent protein diet.

Folic acid and 6-pteridylaldehyde neither increased nor decreased the liver xanthine oxidase in rats when incorporated in purified or natural diets.

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THE USE OF LACTOBACILLUS LEICHMANNII IN THE ESTIMATION OF VITAMIN B₁₂ ACTIVITY*

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Owing to the importance of vitamin B₁₂ in animal nutrition, a convenient method for its estimation in natural materials and foodstuffs is of definite value. Certain lactic acid bacteria have been shown to respond to graded levels of vitamin B₁₂ and related substances (1-3). In the present paper a method for the estimation of the vitamin B₁₂ activity of natural materials through the use of *Lactobacillus leichmannii* is described.

EXPERIMENTAL

Cultures and Inocula—The organism used was *Lactobacillus leichmannii* ATCC 4797. It was carried in litmus milk enriched with 0.1 ml. of Reticulogen (Lilly, 20 U. S. P units per ml.) per liter of milk and transferred biweekly. The cultures were incubated at 37° for 18 to 24 hours and then refrigerated until transferred.

The inoculum broth was prepared by adding Reticulogen (0.1 ml. per liter) or other liver preparations in an equivalent amount to the basal medium. The broth was then placed in test-tubes in 5 ml. amounts and sterilized in an autoclave for 20 minutes at 121°. It was refrigerated until used. Inoculum was prepared by transfer from the litmus milk and incubated at 37° for 18 hours. The bacterial cells were centrifuged, washed, and resuspended in approximately 25 ml. of sterile saline. 1 drop of this dilution, as dispensed from a syringe, was added to each tube (except the uninoculated blank).

Procedure—Assays were set up in 18 × 150 mm. rimless, Pyrex test-tubes. Since small amounts of contaminating vitamin B₁₂ could cause appreciable errors, the tubes were washed in a trisodium phosphate solution after each assay and were thoroughly rinsed and dried before using. Periodically, the glassware used in the procedure was cleaned in a chromic acid solution.

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A standard stock solution of vitamin B₁₂ was prepared by diluting the vitamin B₁₂ in sterile 0.02 M Na₂HPO₄-KH₂PO₄ buffer of pH 6.8.¹ This was stored under toluene in the cold. The standard curve was prepared by adding sufficient diluted stock solution to a series of tubes to give 0.025, 0.05, 0.075, 0.10, 0.25, 0.5, and 0.75 mμgm. of vitamin B₁₂ per tube. Distilled water and double strength medium were added to bring the final volume to 10 ml. In some of the early experiments different concentrations of vitamin B₁₂ were used. Samples were added so as to supply five different levels of the vitamin. A reference standard (a liver preparation)

TABLE I
Composition of Medium

Component	Quantity per 100 ml. double strength medium	Component	Quantity per 100 ml. double strength medium
HCl-hydrolyzed casein	1 gm.	Biotin	1 γ
Tryptophan	20 mg.	Pyridoxine	400 "
Cysteine	20 "	Pyridoxal	400 "
Adenine sulfate	1 "	Riboflavin	200 "
Guanine hydrochloride	1 "	Thiamine	200 "
Uracil	1 "	Calcium pantothenate	200 "
Xanthine	1 "	Nicotinic acid	200 "
Salts A*	1 ml.	Folic acid	100 "
" B*	1 "	Ascorbic acid	200 mg.
Sodium acetate (anhydrous)	1.2 gm.	Fumaric acid	100 "
Glucose	4.0 "	Tween 80	0.2 ml.

* Salts A contain 25 gm. of K₂HPO₄ and 25 gm. of KH₂PO₄ dissolved in 250 ml. of water. Salts B contain 10 gm. of MgSO₄·7H₂O, 0.5 gm. of NaCl, 0.5 gm. of FeSO₄·7H₂O, and 0.5 gm. of MnSO₄·4H₂O dissolved in 250 ml. of water.

was included whenever samples were assayed as a further check on the validity of the observed values.

The tubes (60 per rack) were covered with a metal cover lined with cotton and were autoclaved for 3 minutes at 121°. Then, in a steamed room, 1 drop of inoculum was added to each tube except the blank. In the later phases of the work, in order to insure uniform inoculation, the medium was preinoculated and then dispensed aseptically to the tubes. The assay was read turbidimetrically in an Evelyn colorimeter after 15 to 18 hours incubation in a constant temperature water bath at 37°.

Basal Medium—The composition of the basal medium finally adopted is shown in Table I. It is a modification of the medium of Skeggs *et al.*

¹ We are indebted to Merck and Company, Inc., Rahway, New Jersey, for crystalline vitamin B₁₂.

(4) and was selected after determining assay curves for various modifications of the original medium. Growth response, linearity over the assay range, and low blanks were used as criteria for selecting the medium.

Preliminary studies with the medium of Skeggs *et al.* (4) showed that the tryptic digest of casein could be omitted if clarified tomato juice was included. Table II shows the growth response when 0.5 ml. of clarified tomato juice and 2 mg. of ascorbic acid were added per 10 ml. of medium, instead of the tryptic digest of casein. Since the trypsin in our laboratory showed appreciable vitamin B₁₂ activity, the omission of the enzymatic casein was desirable, for it lowered the blank and greatly increased the useful assay range. Although this modified medium appeared suitable for assay of vitamin B₁₂ activity, considerable variability in assay values

TABLE II

Effect of Omitting Tryptic Digest of Casein and Adding Tomato Juice and Ascorbic Acid on Response of L. leichmannii 4797 to Vitamin B₁₂

Concentration of vitamin B ₁₂ per tube	Galvanometer reading	
	Enzymatic casein	Tomato juice and ascorbic acid
<i>μgm.</i>		
0	81	98
0.1	73	82
0.2	66	70
0.4	55	53
0.6	46	43
0.8	42	41
1.0	39	40

was observed. This variability appeared to be related to the quality of the clarified tomato juice.

However, before studying the medium further, the culture was examined to see whether contamination had occurred. The culture was plated by a dilution method. Well over 99 per cent of the colonies exhibited characteristics of the lactic acid organisms as viewed under a microscope. A number of the colonies were isolated and all responded identically to known concentrations of vitamin B₁₂. However, growth response was still inadequate on the tomato juice-ascorbic acid medium.

Therefore, the basal medium used in the following study was the medium of Skeggs *et al.* (4) modified by omitting the enzymatic digest of casein. It has been reported that sodium thioglycolate added to a medium similar to the one under study protected vitamin B₁₂ against destruction during autoclaving (5). Accordingly, sodium thioglycolate and tomato juice were compared. Ascorbic acid and cysteine were also studied. The

results are shown in Table III. Cysteine and ascorbic acid stimulated growth markedly. Sodium thioglycolate appeared inferior. However, the poor results may have been due to heavy metal contamination. When the sodium thioglycolate was taken up in ether and finally recrystallized from benzene, the growth response was nearly equal to that produced by ascorbic acid.

TABLE III

Effect of Adding Cysteine, Sodium Thioglycolate, Ascorbic Acid, and Clarified Tomato Juice on Response of L. leichmannii 4797 to Vitamin B₁₂

Concentration of vitamin B ₁₂ per tube	Galvanometer reading			
	Cysteine, 1.3 mg. per tube	Sodium thioglycolate, 1.3 mg. per tube	Autoclaved tomato juice, 0.5 ml. per tube	Ascorbic acid, 2 mg. per tube
<i>μgm.</i>				
0	99	99	97	98
0.2	63	80.5	93	64
0.4	50.5	67	78	53
0.6	46	61	67	48
0.8	44	58	57	46
1.0	42	55	47	42
2.0	37.5	50	43	41

TABLE IV

Effect of Adding Fumarate on Response of L. leichmannii to Vitamin B₁₂

Concentration of vitamin B ₁₂ per tube	Galvanometer reading	
	Cysteine	Cysteine and fumaric acid
<i>μgm.</i>		
0	98	99
0.03	89	91
0.06	82	80
0.09	77	75
0.15	71	68
0.30	60	53
0.60	48	42

Riesen *et al.* (6) have shown that cysteine can replace cystine for certain lactic acid bacteria. In this study when cysteine replaced rather than supplemented the cystine the growth response appeared to be slightly superior. The results were, however, of doubtful significance.

The effect of fumarate was studied by running standard curves with and without fumaric acid. The results (Table IV) showed that fumaric acid gave an additional response, and it was therefore included in the basal media.

By use of the medium in Table I, recovery values of 88 to 110 per cent have been obtained with several liver preparations. Reproducibility of ± 15 per cent has been observed with reference samples.

Analysis of Animal Proteins—A variety of protein materials has been analyzed for vitamin B₁₂ activity with the medium shown in Table I. Because water extracts of beef muscle failed to give values comparable to those obtained with animal assays (7), various methods of releasing the substance (or substances) responsible for the vitamin B₁₂ activity were tried. A 24 to 30 hour digestion of homogenized sample with trypsin in

TABLE V
Vitamin B₁₂ Activity of Some Animal Materials

Animal material	Vitamin B ₁₂ activity per 100 gm. dry weight
	γ
Beef round (cooked).....	5.0
“ shoulder (cooked).....	3.9
“ loin (cooked).....	3.0
“ tongue (cooked).....	7.6
“ kidney “.....	42.0
Pork shoulder (cooked).....	5.0
“ hocks (cooked).....	0.1
“ loin (cooked).....	3.0
“ ham “.....	2.9
Veal (cooked).....	2.0
Leg of lamb (cooked).....	8.0
Horse meat (canned).....	7.0
Beef liver (fresh).....	47.0
“ heart “.....	9.5
“ shoulder (lyophilized).....	4.9
Leg of lamb “.....	9.6
Casein (alcohol-extracted).....	0.2

an 0.8 per cent sodium bicarbonate solution was finally selected, as it appeared to give the maximum release. Digestion near the neutral point was found to be essential. Autolysis did not give full release of activity or else resulted in a destruction of activity. Pancreatin was found to be satisfactory, but its use resulted in extremely high blanks.

Tryptic digests of a number of meat samples have been analyzed for vitamin B₁₂ activity. The values are presented in Table V and represent the average of at least two determinations of five tubes each. It should be stressed that the values presented do not represent absolute vitamin B₁₂ concentration since substances other than vitamin B₁₂ have been shown to be active for the test organism (4, 8).

SUMMARY

1. A microbiological assay for vitamin B₁₂ activity for *Lactobacillus leichmannii* ATCC 4797 has been developed. Satisfactory standard curves, low blanks, and satisfactory recovery values were obtained.
2. A method for the release of vitamin B₁₂ activity from animal material has been described.
3. The vitamin B₁₂ activity of various animal materials has been presented.

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SOME EFFECTS OF RESTRICTION OF DIETARY PROTEIN ON THE INTRACELLULAR COMPONENTS OF LIVER*

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The effect of restriction of dietary protein on the chemical composition of the liver has received considerable attention in recent years. While this organ as a whole has been found to be profoundly influenced by protein depletion of the animal, little information has appeared in the literature concerning the effects of such an experimental procedure on the composition of the various morphological components of the liver cell *per se*. With the advent of relatively simple centrifugation techniques for the separation of liver cells into nuclear, mitochondrial, microsomatic, and residual cytoplasmic components (1, 2), it is now feasible to investigate the specific intracellular fractions as they are affected by restriction of dietary protein. As an initial effort in this direction, the present study was undertaken to determine the site of changes occurring in the liver cells of protein-depleted rats with respect to the concentrations of nitrogen, total phosphorus, nucleic acids, and riboflavin. These chemical constituents were chosen for experimental consideration since the manner in which their concentrations are influenced in the whole liver has been previously investigated.

EXPERIMENTAL

Twenty female rats of the Wistar strain, between 15 and 17 weeks of age and weighing between 170 and 234 gm., were placed on a normal diet, adequate in all respects, for a period of 7 days. At the end of this time ten of the animals were placed on a protein-free diet for a period of 21 days. The remaining ten animals served as controls and were continued on the normal diet for 21 days.

The normal diet consisted of commercial casein 24 per cent, glucose 63 per cent, corn oil 8 per cent, Salts 4 (3) 4 per cent, and dried brewers' yeast 1 per cent. The protein-free diet was similar in composition except that the casein was replaced by an equal amount of glucose. The following vitamins were incorporated into each 100 gm. of diet in the amounts indi-

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cated: thiamine 0.2 mg., nicotinic acid 2.5 mg., riboflavin 0.3 mg., calcium pantothenate 4 mg., pyridoxine hydrochloride 0.5 mg., inositol 10 mg., choline chloride 100 mg., and 2-methylnaphthoquinone 1 mg. Twice weekly each animal received 2 drops of a cod liver oil-vitamin E supplement which contained 200 U. S. P. units of vitamin A, 20 U. S. P. units of vitamin D, and 1 mg. of α -tocopherol.

At the end of its feeding period each animal was sacrificed by a sharp blow on the head. The entire liver was quickly excised and blotted free of adhering blood. In all cases the left lateral lobe was placed in ice-cold isotonic KCl solution and used for the preparation of a homogenate as described below. The remainder of the liver was sampled for the determination of water and fat. Water was determined by drying the liver sample to constant weight at 103°, and fat by weight difference after extraction with ether.

The left lateral lobe was removed from the KCl, blotted on filter paper, and a portion weighing exactly 1500 mg. was homogenized in cold 8.5 per cent (isotonic) sucrose solution in a Potter-Elvehjem glass homogenizer (4). The homogenate was diluted to 15 ml. with cold isotonic sucrose solution, and a 10 ml. aliquot was fractionated into so called nuclear, mitochondrial, microsomatic, and residual cytoplasmic fractions by a method similar to that described by Schneider (2). All centrifugations for the preparation of these fractions were performed in an International refrigerated centrifuge, model PR-1, at 2°, and with 20 ml. Lusteroid tubes. When indicated, washing of the sedimented fractions was carried out with 2.5 ml. portions of cold isotonic sucrose solution under the same conditions of time and speed of centrifugation as in the original sedimentation of the given fraction.

The nuclear fraction was obtained by centrifugation at $200 \times g^1$ for 10 minutes with the horizontal head No. 269. The supernatant was decanted and the sedimented material was resuspended carefully and washed twice. The washed fraction was dispersed evenly and diluted to 3 ml. with isotonic sucrose and as such was used for subsequent analyses.

The washings from the nuclear fraction were combined with the original supernatant and the mixture subjected to further centrifugation at $8500 \times g$ for 10 minutes with the multispeed attachment and head No. 296. The sediment was washed twice and diluted to a volume of 3 ml. with sucrose solution. This material constituted the mitochondrial fraction used in the subsequent analyses.

The supernatant fluid and washings from the mitochondrial fraction were combined and the mixture further centrifuged at $20,000 \times g$ for 1 hour, again with head No. 296. The resulting supernatant was decanted and

¹ Centrifugal forces were calculated for the center of the tubes employed.

diluted to 20 ml. with sucrose solution, and as such constituted the so called residual cytoplasmic fraction. The sedimented material was suspended in sucrose solution to a volume of 3 ml. and represented the microsomatic fraction.

In one respect the procedure outlined above for the preparation of the liver cell fractions differs from that employed by Schneider (2). In the method described by this worker a gravitational force of $600 \times g$ was used for the sedimentation of the nuclear fraction. It was found, however, that with the centrifugation equipment utilized in the present study a gravitational force of this magnitude resulted in the separation of a fraction which contained considerably more nitrogen than has been reported for the nuclear fraction, and which, upon staining, showed a relatively high percentage of mitochondria. Accordingly, a comparison was made of the morphological characters of fractions prepared from the same homogenate by the use of forces of 200, 400, and $600 \times g$, respectively. It was found that only the material sedimented at $200 \times g$ was relatively free of mitochondria, and in addition the supernatant decanted from this sediment showed only a few nuclei upon staining. The nitrogen content of the nuclear fraction prepared in this manner compared favorably with figures given in the literature. For these reasons a force of $200 \times g$ was routinely used for the separation of nuclear fractions.

Some difficulty was experienced in the preparation of the mitochondrial fraction. The material obtained in the first centrifugation at $8500 \times g$, when washed and recentrifuged, separated into a firmly packed sediment and a material which layered loosely on the surface of the sediment and could be easily decanted. Consequently, when the wash fluid was completely decanted, this material appeared in the microsomatic fraction. Examination of stained preparations of the loosely layered material showed numerous cell fragments but revealed the presence of relatively few mitochondria. For this reason, the loose layer was considered to belong with subsequent fractions, and therefore, in the preparation of fractions to be used for chemical analysis, was routinely decanted from the mitochondria. It must be pointed out, however, that, while mitochondrial and microsomatic fractions prepared in this manner yielded pentosenucleic acid (PNA) values closely approximating those reported by Schneider (2), the nitrogen content of the mitochondrial fraction was appreciably lower and that of the microsomatic fraction appreciably higher than the corresponding values obtained by this investigator. Because of this difference in nitrogen distribution, fractions were prepared in which the loosely layered material was retained with the mitochondria. When this procedure was followed, the nitrogen contents of the mitochondrial and microsomatic fractions were in agreement with those reported by Schneider, while the PNA value for

the mitochondria was considerably greater and that of the microsomes was considerably less than the corresponding values given in the literature (2).

The four fractions described above and the original homogenate were analyzed for desoxypentosenucleic acid (DNA), pentosenucleic acid, riboflavin, nitrogen, and phosphorus. The desoxypentosenucleic acid was determined by the diphenylamine reaction (5), and the pentosenucleic acid by the orcinol reaction (6) with appropriate corrections in the calculations for the interference of DNA with this determination. The nucleic acid analyses were carried out on trichloroacetic acid extracts prepared according to the method of Schneider (7) without removal of phospholipides. The riboflavin was determined by the fluorometric method of Scott and coworkers (8), adapted to the measurement of micro quantities. Nitrogen was determined by the micro-Kjeldahl procedure, the ammonia formed being steam-distilled and titrated. The phosphorus was estimated by the method of Gomori (9).

Results

The average body weight at sacrifice of the animals maintained on the protein-free diet was 165 gm. and that of the animals on the control diet 204 gm. This difference of weight was a reflection of an average weekly loss of 14 gm. of body weight in the case of the protein-deficient group and an average weekly gain of 6 gm. in the case of the control animals. The average liver weight at sacrifice of the deficient group was 5.22 gm., while that of the control group was 6.63 gm. The changes encountered in the water and fat contents of the livers of the protein-restricted animals compared with the control animals were similar to those reported previously (10).

The more specific chemical changes encountered are presented below.

Nitrogen—The results obtained from the analysis for nitrogen of the homogenate and the individual fractions are given in Table I. As Table I shows, the maintenance of rats on a protein-free diet resulted in a significant decrease in the concentration of nitrogen per gm. of liver. Thus the livers of the protein-deficient rats contained, on the average, 5.76 mg. less nitrogen per gm. than did the livers of the control rats. The nuclear fraction did not contribute to this difference in nitrogen values, whereas the microsomatic fraction accounted for 47.4 per cent, the residual cytoplasmic material for 30.9 per cent, and the mitochondrial fraction for 20.0 per cent of the loss of nitrogen. The actual percentage of nitrogen lost from each fraction, when values from control rats were used as the basis for comparison, were for whole liver 19.3 per cent, nuclear fraction 0 per cent, mitochondrial fraction 22.4 per cent, microsomatic fraction 34.4 per cent, and residual cytoplasm 13.7 per cent.

Phosphorus—The phosphorus values obtained in the analysis of the rat livers and their separate fractions are given in Table I. Of the changes found in the phosphorus concentrations of the various fractions of the livers

TABLE I
Nitrogen and Phosphorus Contents of Liver Cell Fractions from Control and Protein-Deficient Rats

	Nitrogen					Phosphorus				
	Control		Protein-deficient		<i>t</i> value*	Control		Protein-deficient		<i>t</i> value
	Average	$\sigma^\dagger$	Average	σ		Average	σ	Average	σ	
Homogenate										
Mg. per gm. liver.....	29.78	1.03	24.02	2.31	7.21	3.34	0.11	3.13	0.29	2.02
Nuclear fraction										
Mg. per gm. liver.....	4.54	0.35	4.56	0.76	0.08	0.54	0.05	0.55	0.10	0.27
% of total.....	15.3	1.4	18.9	1.6	5.35	16.0	2.1	17.5	2.7	1.32
Mitochondrial fraction										
Mg. per gm. liver.....	5.14	0.77	3.99	0.48	4.01	0.40	0.05	0.36	0.06	1.53
% of total.....	17.5	2.2	16.5	1.8	1.11	11.9	1.3	11.5	1.8	0.54
Microsomatic fraction										
Mg. per gm. liver.....	7.95	0.53	5.22	0.73	9.58	1.18	0.07	0.90	0.07	8.60
% of total.....	26.8	2.4	21.7	2.2	4.95	35.3	2.2	29.0	3.0	5.07
Residual cytoplasmic fraction										
Mg. per gm. liver.....	12.95	0.46	11.17	1.14	4.58	1.56	0.06	1.63	0.17	1.16
% of total.....	43.6	1.1	46.6	2.5	3.47	46.9	2.6	52.1	4.5	3.01
Per cent recovery										
	103.2	2.0	103.8	2.4		110.4	4.2	110.2	5.1	

* The *t* value is the expression of the significance of the difference between the control and protein-deficient values. A figure of 3 or greater is of definite significance (16).

$\dagger \sigma$ represents the standard deviation.

of the protein-deficient animals compared with those of the control animals, the most noteworthy was a decrease in the phosphorus of the microsomatic fraction. Although statistical analysis showed the changes in the

other fractions to be of doubtful significance, they were nevertheless in the same direction as the nitrogen changes.

Desoxypentosenucleic Acid—As is shown in Table II, the livers of the

TABLE II

Desoxypentose nucleic and Pentosenucleic Acid Contents of Liver Cell Fractions from Control and Protein-Deficient Rats

For explanation of *t* value and σ see foot-note to Table I.

	Desoxypentosenucleic acid					Pentosenucleic acid				
	Control		Protein-deficient		<i>t</i> value	Control		Protein-deficient		<i>t</i> value
	Average	σ	Average	σ		Average	σ	Average	σ	
Homogenate										
Mg. per gm. liver.....	2.68	0.21	3.06	0.40	2.68	8.39	0.17	8.03	1.02	1.05
" " " " N.....	90.3	7.7	128	16	6.78	282	15	334	38	3.84
Nuclear fraction										
Mg. per gm. liver.....	2.52	0.21	3.26	0.40	5.10	1.13	0.20	0.96	0.18	1.91
% of total.....	93.7	6.9	107.5	14.9	2.63	13.5	3.0	12.0	2.3	0.40
Mg. per gm. fraction N.....	559	57	718	66	5.63	247	44	208	17	2.48
Mitochondrial fraction										
Mg. per gm. liver.....						0.43	0.08	0.56	0.15	2.32
% of total.....						5.2	0.9	6.9	1.4	3.16
Mg. per gm. fraction N.....						84	16	141	33	4.70
Microsomatic fraction										
Mg. per gm. liver.....						4.16	0.60	3.49	0.31	5.07
% of total.....						55.1	7.0	43.8	4.8	4.28
Mg. per gm. fraction N.....						580	58	688	88	3.13
Residual cytoplasmic fraction										
Mg. per gm. liver.....						3.04	0.49	3.78	0.69	2.67
% of total.....						36.2	5.5	46.9	4.7	4.68
Mg. per gm. fraction N.....						234	35	337	48	5.33
Per cent recovery										
	93.7	6.9	107.5	14.9		110.1	8.9	109.7	6.2	

protein-deficient animals contained on the average more desoxypentose-nucleic acid per gm. of tissue than did the livers of the control animals. This difference was also seen in the nuclear fraction, a finding which is con-

sistent with the fact that this fraction has been shown to contain all of the DNA. Statistical analysis of the data shows that this increase was of probable, although not conclusive, significance.

Pentosenucleic Acid—Table II contains the results of the analyses for pentosenucleic acid. These data show that, although the livers of both sets of animals had approximately the same total amount of pentosenucleic acid per gm. of tissue, there was a marked change from the controls in the distribution of PNA among the various fractions obtained from the pro-

TABLE III

Riboflavin Content of Liver Cell Fractions from Control and Protein-Deficient Rats

For explanation of *t* value and σ see foot-note to Table I.

Fraction	Riboflavin				
	Control		Protein-deficient		<i>t</i> value
	Average	σ	Average	σ	
Homogenate, γ per gm. liver.....	26.93	3.02	17.06	1.64	8.58
“ mg. per gm. liver <i>N</i>	0.90	0.09	0.72	0.08	4.45
Nuclear, γ per gm. liver.....	2.89	0.38	2.21	0.42	3.60
“ % of total.....	11.0	2.1	13.0	2.4	1.88
“ mg. per gm. fraction <i>N</i>	0.63	0.07	0.49	0.10	5.90
Mitochondrial, γ per gm. liver.....	10.47	2.13	7.40	1.01	3.90
“ % of total.....	38.7	5.7	43.9	7.8	1.62
“ mg. per gm. fraction <i>N</i>	2.03	0.23	1.88	0.26	1.29
Microsomatic, γ per gm. liver.....	7.62	1.49	4.28	.10	5.41
“ % of total.....	29.1	8.1	25.2	16.4	1.13
“ mg. per gm. fraction <i>N</i>	0.96	0.19	0.80	0.16	1.14
Residual cytoplasmic, γ per gm. liver.....	6.98	1.43	5.92	0.96	1.85
“ “ % of total.....	26.2	6.4	35.3	7.0	2.88
“ “ mg. per gm. fraction <i>N</i>	0.54	0.12	0.54	0.12	0
Recovery, %.....	105.0	13.5	117.4	11.4	

tein-deficient rats. This change was particularly evident in the microsomatic and residual cytoplasmic fractions in the protein-deficient group, the former fraction containing 43.8 per cent of the total PNA compared with 55.1 per cent in the same fraction of the control series, and the cytoplasmic fraction containing 46.9 per cent of the total PNA compared with 36.2 per cent in the control animals. The apparent redistribution of pentosenucleic acid from the microsomatic to the residual cytoplasmic fraction paralleled the changes in phosphorus and nitrogen content of these fractions.

Riboflavin—As shown in Table III, each gm. of liver from the protein-

deficient rats contained on the average less riboflavin than did a gm. of liver from the control rats. This change was a reflection of a proportionate decrease of riboflavin in all of the fractions analyzed; hence the percentage distribution of the total riboflavin among the various fractions was not significantly different in the protein-deficient and control animals.

DISCUSSION

Campbell and Kosterlitz (11) demonstrated that the livers of rats maintained on a protein-free diet for 1 week showed an increase in DNA content and a diminution in PNA content compared with corresponding control animals. While the results reported in the present paper, which were obtained with rats fed a protein-free diet for 3 weeks, show changes similar in direction, statistical analysis of the data presented here indicates that the increase of DNA was of probable significance, while the decrease of PNA was of questionable significance. It is of interest that fasting, like restriction of dietary protein, also affects the nucleic acid content of the liver, the change, however, being due to a loss of pentosenucleic acid (12, 13).

With regard to the constituents in the various liver cell components, the results would seem to indicate that the most dramatic effects of protein restriction occurred in the microsomatic fraction. In this fraction the PNA decreased from 55.1 to 43.8 per cent of the total liver PNA, the phosphorus decreased from 35.3 to 29.0 per cent of the total, and the nitrogen from 26.8 to 21.7 per cent. In each case the decrease was concomitant with a comparable increase of the given constituent in the residual cytoplasmic fraction. Thus the PNA in the residual cytoplasm increased from 36.2 to 46.9 per cent of the total liver PNA, the phosphorus from 46.9 to 52.1 per cent, and the nitrogen from 43.6 to 46.6 per cent. The combined percentage values in the microsomatic and residual cytoplasmic fractions for each of these three constituents were the same in both the control and protein-deficient groups, indicating that a shift had occurred between the two fractions. This apparent redistribution may be interpreted in either of two ways: (a) that the amount of "new" protein and PNA entering the microsomes (*e.g.*, by synthesis or exchange) did not exceed the rate of loss of these constituents from this fraction, whereas the amount of these substances entering the residual cytoplasmic fraction exceeded their loss; or (b) that there was an absolute decrease in the number of microsomatic particles occasioned by dissolution of some of them into the cytoplasmic material. Although there is no direct experimental evidence bearing on this point, the latter hypothesis seems more reasonable.

In contrast to the lability of the microsomatic fraction, the relative constancy of the nitrogen, phosphorus, and PNA concentrations of the nuclear

fraction would seem to indicate that this cellular component is less readily affected by protein depletion of the animal, a view which is held by Kosterlitz (14). However, if one accepts the view of Mirsky and Ris (15) that nuclei, under various conditions, contain the same amount of DNA, then the increase of this constituent per gm. of liver could only be accounted for by an increase in the number of nuclei (*i.e.*, cells) in the sample. From this it would follow that the nuclei actually contained less nitrogen, phosphorus, and PNA in the case of the protein-free animals, but the decrease was masked in the analysis by a concomitant increase in the number of nuclei per sample.

The results further show that the so called labile protein of the liver cell is not restricted to any single morphological entity, inasmuch as the mitochondrial, microsomatic, and residual cytoplasmic fractions all exhibited a loss of nitrogen following protein restriction. Of these fractions, the microsomatic was most affected by protein depletion, as demonstrated by the fact that it "lost" approximately 35 per cent of its original nitrogen. This, in fact, represented about 50 per cent of the total nitrogen lost by the liver.

It is noteworthy that, while the nuclear fraction did not decrease in nitrogen content as a result of protein depletion of the animals, the riboflavin content of this fraction decreased by 23.5 per cent of the control value. Thus, its riboflavin content was not related to its nitrogen content, in contrast to the direct relationship found previously between these constituents in the whole liver (10). This relationship was found to hold, however, for the other liver cell fractions.

SUMMARY

The effects of feeding rats a diet free of protein for a period of 21 days have been studied with respect to the distribution of nitrogen, phosphorus, nucleic acids, and riboflavin among the nuclear, mitochondrial, microsomatic, and residual cytoplasmic fractions of the liver cell.

Protein deficiency was accompanied by a decrease in the concentration of liver nitrogen, which was the result of a loss of nitrogen from all separated fractions of the liver cell except the nuclear. While the total phosphorus content of the whole liver was not affected significantly by depletion of dietary protein, there was a redistribution of phosphorus from the microsomatic fraction to the residual cytoplasm.

Protein restriction resulted in an increase in the desoxypentose nucleic acid content of the liver while causing relatively little change in the pentose nucleic acid content. The percentage of the total liver PNA located in the microsomatic fraction was decreased concomitantly with an increase in the percentage of total liver PNA in the residual cytoplasmic fraction.

Protein-depleted rats exhibited a decrease in the concentration of riboflavin in their livers compared with control rats. This change was the result of a decrease of riboflavin in all of the cellular fractions analyzed.

The results would seem to indicate that, of the liver cell components studied, the microsomatic fraction was most affected by the restriction of dietary protein.

The authors are indebted to Professor Leonard G. Worley of Brooklyn College for the histological examination of the various liver cell fractions.

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THE EFFECT OF DIFFERENT DOSE LEVELS OF ZIRCONIUM ON THE EXCRETION AND DISTRIBUTION OF PLUTONIUM AND YTTRIUM

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Early administration of zirconium citrate to rats previously injected with tracer levels of plutonium, Pu^{239} , and yttrium, Y^{91} , results in a marked increase in the urinary excretion and a considerable decrease in the bone content of the injected radioelements (1, 2). Relatively large amounts of zirconium, about 200 mg. per kilo, were used in these experiments. In order to clarify the mechanisms involved, graded dose levels ranging from 12.3 to 1336 mg. per kilo as Zr have been given.

EXPERIMENTAL

Fifteen Sprague-Dawley female rats with weights ranging from 183 to 197 gm. were given a single intraperitoneal injection of tetravalent plutonium and trivalent yttrium contained in a 1 per cent sodium citrate solution at pH 6. Each rat received about 17 γ of Pu^{239} , equivalent to 1.2×10^6 c.p.m., and about 18×10^3 c.p.m. of Y^{91} . 1 hour after receiving the Pu and Y, a group of five rats was given a single intraperitoneal injection with a zirconium citrate solution (4) containing 9.85 mg. of Zr per cc. Another group of four rats was injected at the same time and then with five additional doses of zirconium at hourly intervals. The total mg. per kilo of Zr received by each rat is shown in Tables I and II. All treated rats were sacrificed 72 hours after the Pu and Y injection. The six control rats, receiving only the radioelements, were sacrificed in pairs at 1, 6, and 72 hours after injection. All the rats were kept in individual metabolism cages and the urine and feces collected separately. The liver, one femur, a blood sample, and the carcass, as well as the urine and feces, were analyzed for their radioelement content. The total skeletal and blood contents were estimated.¹ Experimental procedures and methods of assay were identical to those previously described (4).

RESULTS AND DISCUSSION

Considering first the effects of zirconium on plutonium metabolism, it is seen from Fig. 1 that the amount of Pu excreted into the urine was pro-

¹ The total blood volume in cc. was estimated from Donaldson's tables (3) to be 0.0606 times the body weight in gm. The amount of Pu or Y in the entire skeleton was calculated, somewhat arbitrarily, to be 20 times that in a single femur.

portional to the size of the dose of zirconium;² the reduction in skeletal content was nearly independent of the amount of Zr injected or of the number of doses of Zr given; and liver retention was reduced at the higher dose levels of Zr, but increased following small doses. From Table I it is further seen that the blood level of Pu was significantly reduced, even 3 days after the Zr injection, and that some Pu was removed from the carcass of those rats receiving more than 105 mg. per kilo of Zr.

At the time the Zr injections were initiated, the blood contained about 39 per cent of the injected dose of Pu, the liver 13 per cent, and the skeleton 15 per cent (Table I). When these data are considered together

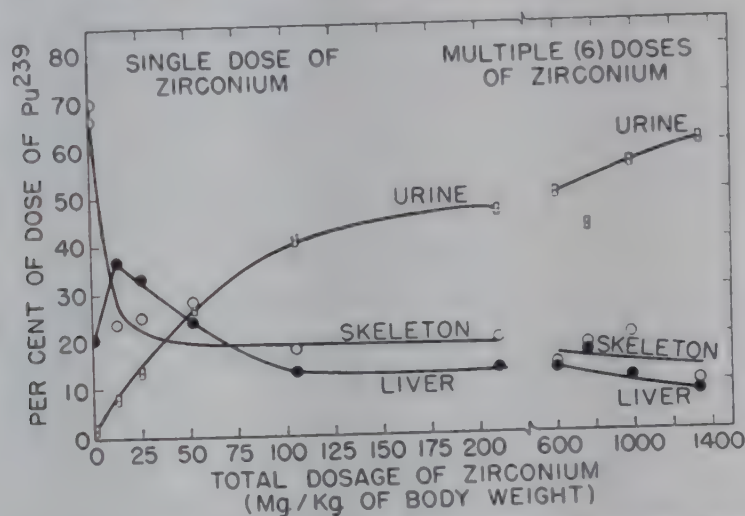


FIG. 1. The effect of intraperitoneally administered zirconium citrate on the distribution of plutonium in the urine and tissues. Zirconium injections were begun 1 hour after intraperitoneal injection of Pu^{239} and all the rats were sacrificed at 72 hours.

with the distribution and excretion of Pu in the treated rats, it appears that the gross mode of action of Zr upon the metabolism of Pu was as follows: (a) Nearly all of the Pu was removed from the blood immediately following the administration of Zr. Some of this Pu was retained in the liver and some excreted by the kidneys, the relative amounts depending upon the dose of Zr; when the dose of Zr was less than 52 mg. per kilo some was retained in the liver, but when the dose was greater than 105 mg. per kilo, all the circulating Pu was excreted. (b) Since the femur content of the treated animals was essentially the same as that of the 1 hour controls, no Pu was actually removed from the skeleton under these experimental conditions; rather, further deposition was prevented by removal of the circulating plutonium. (c) The Pu removed from the car-

² Nearly all (>95 per cent) of the increased urinary excretion of Pu or Y observed following Zr administration took place during the subsequent 24 hours.

TABLE I
*Distribution and Excretion of Injected Plutonium in Rats As Function of Zirconium Administered**

The figures are expressed as per cent of injected dose, adjusted to 100 per cent recovery. The average recovery was 99.3 ± 5 per cent of the injected dose.

Organ	In control animals			In zirconium-treated animals									
	1 hr.	6 hrs.	72 hrs.	Single injection, total mg. Zr per kilo					Multiple injections, total mg. Zr per kilo				
				12.3	24.9	52.1	105	210	603	763	985	1336	
Carcass less blood.....	46.9	67.4	70.9	52.3	47.9	45.2	41.7	36.4	34.2	38.0	30.4	24.2	
Femur.....	0.75	1.49	3.42	1.19	1.26	1.41	0.90	0.99	0.73	0.93	1.02	0.62	
Liver.....	13.2	10.0	20.7	36.8	33.3	23.9	13.3	13.5	13.3	16.7	11.4	8.06	
Urine total.....	0.3	0.6	1.32	8.12	13.8	26.8	40.7	46.4	50.0	42.9	55.5	60.6	
Feces ".....	0.0	0.1	3.06	1.57	3.66	2.53	3.33	2.86	1.74	1.61	1.51	6.53	
Blood " †.....	38.9	20.5	0.6	0.03	0.04	0.14	0.1	0.04	0.03	0.04	0.03	0.02	

* All the rats, except the 1 and 6 hour controls, were killed 72 hours after receiving the plutonium. Each group in the Zr-treated animals is represented by one rat, while in the controls the value for each group is the average from two rats.

† The blood volume was taken to be 6.06 per cent of the body weight (3).

TABLE II
*Distribution and Excretion of Injected Yttrium in Rats As Function of Amount of Zirconium Administered**

The figures are expressed as per cent of injected dose, adjusted to 100 per cent recovery. The actual recovery was 95.1 ± 6 per cent of the injected dose.

Organ	In control animals			In zirconium-treated animals								
	1 hr.	6 hrs.	72 hrs.	Single injection, total mg. Zr per kilo				Multiple injections, total mg. Zr per kilo				
				12.3	24.9	52.1	105	210	603	763	985	1336
Carcass less blood.....	74.9	67.0	62.4	58.3	59.8	52.2	54.1	49.3	42.4	45.6	38.7	28.8
Femur.....	1.90	2.41	2.35	2.20	2.32	1.97	1.76	1.89	1.80	1.60	1.59	1.13
Liver.....	8.14	8.98	4.86	5.83	5.78	4.40	4.30	3.49	2.84	3.02	2.50	1.99
Urine total.....	8.62	19.9	26.6	31.5	26.6	38.6	35.3	41.9	51.5	47.7	56.0	61.0
Feces “.....	0.0	0.39	3.82	2.27	5.51	2.91	4.55	3.41	1.45	2.07	1.25	7.09
Blood “ †.....	6.5	1.5	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3

* All the rats, except the 1 and 6 hour controls, were killed 72 hours after receiving the yttrium. Each group in the Zr-treated animals is represented by one rat, while in the controls the value for each group is the average from two rats.

† The blood volume was taken to be 6.06 per cent of the body weight (3).

cass of rats receiving the larger dosages of Zr was derived from the soft tissue components, primarily the muscle.

At the time of injection of Zr, only 6.5 per cent of the injected Y was in the circulating blood (Table II). Hence, the net increase in the amount of Y in the urine as well as the net reduction in skeletal content (Fig. 2) was not as marked as in the case of plutonium. However, at dosages of 210 mg. of Zr per kilo and greater, the increased amount of Y excreted by the kidneys exceeded by several fold the amount present in the blood at the time Zr administration was begun. This additional Y was derived from the liver, the soft tissue components of the carcass, and from the skeleton (probably the marrow).

The fact that a relatively small amount of Zr is needed to clear the

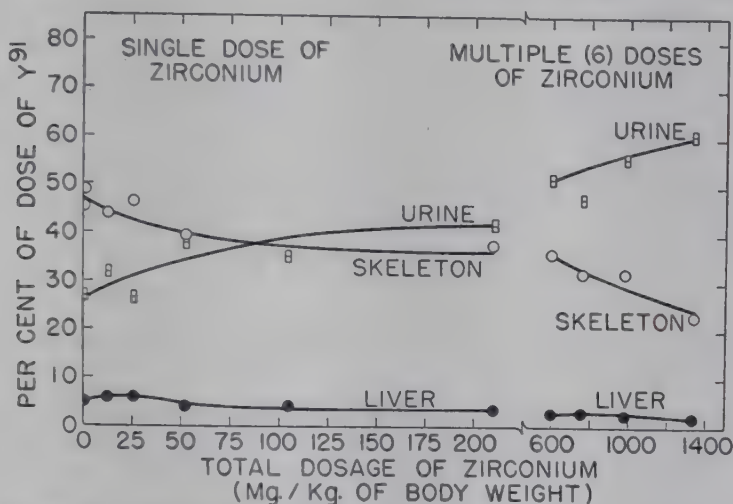


FIG. 2. The effect of intraperitoneally administered zirconium citrate on the distribution of yttrium in the urine and tissues. Zirconium injections were begun 1 hour after intraperitoneal injection of Y^{91} and all the rats were sacrificed at 72 hours.

blood of circulating Pu would indicate that the injection of a certain minimum amount of Zr several days prior to the administration of Pu should act to minimize the deposition of Pu in the skeleton. We have, indeed, found this to be the case.

A means of treating chronic poisoning by those radioelements affected by Zr is suggested by these results. Assuming that the rate of release of a radioelement is proportional to its blood concentration, administration of Zr would be made each time the blood level of the radioelement rose to a plateau following the preceding Zr injection.

SUMMARY

Adult female Sprague-Dawley rats were injected intraperitoneally with a citrate solution containing both Pu^{239} and Y^{91} . Starting 1 hour later,

they were given either one or six intraperitoneal injections of Zr citrate in graded dose levels. They were killed 3 days later.

The amount of Pu or Y excreted in the urine was proportional to the size of the dose of Zr. The reduction of skeletal content of Pu was nearly independent of the amount of Zr injected, but that of Y was increased with increasing dosages of Zr. Liver retention of Pu was increased, the decreased with increasing dosages of Zr, but the Y content of the liver was little affected. 3 days after the injection the blood level of Pu in the Zr-treated rats was much lower than the control level. The amount of radioelement excreted in excess of that removed from the blood was derived in the case of Y from both the skeleton and the other tissues of the carcass, while in the case of Pu it was derived only from the soft tissues excluding the skeleton.

It is suggested that the lowered bone content of Pu which follows the Zr treatment is brought about by very rapid removal of Pu from the blood. This Pu, which otherwise would be deposited mainly in the bone is either excreted by the kidneys or in part deposited in the liver, depending on the amount of Zr. It is probable, however, that prolonged Zr treatments could effect actual removal from bone.

The technical assistance of Rosie Hunter is gratefully acknowledged.

Addendum—Our recent experiments have shown that the minimum amount of Zr which will lessen the skeletal deposition of Pu is between 5.1 and 12.3 mg. per kilo. Following intravenous or intraperitoneal injection of Zr, the blood level of Pu is reduced to one-half the control level within the first 5 minutes, and to one-tenth the control level within 90 minutes. Colloidal solutions of Zr citrate are much more effective in minimizing bone deposition of Pu than the solution employed in this paper. It now appears that Zr (and salts of other hydrolyzable elements such as titanium, iron, and manganese) remove Pu from blood by adsorption of the circulating Pu on the colloidal aggregates which are necessarily formed by the injected Zr in the blood stream.

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A PHOTOMETRIC NINHYDRIN METHOD FOR THE MEASUREMENT OF PROTEOLYSIS*

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The method, here presented, is based on the findings of Moore and Stein (1) who have shown that, over a broad range, the color produced by the interaction of an improved ninhydrin reagent with a large number of amino acids and peptides conforms with Beer's law, although all amino acids and peptides do not produce *equal* color yields.

This procedure has been found to be relatively simple, reasonably accurate, and is especially useful when large numbers of analyses are required. It theoretically may be employed to measure the enzymic hydrolysis of any synthetic peptide.

From the work of Moore and Stein (1), the measurement of the degree of splitting of a peptide bond of a synthetic substrate may be deduced. Since that portion of the synthetic substrate which is hydrolyzed is equal to each of the quantities of the hydrolytic products liberated, the color yield of a sample drawn during the course of enzymic hydrolysis may be represented as follows:

$$m_0(a_0 - a) + m_1a + m_2a = D_x \quad (1)$$

In this equation, a_0 is the initial substrate concentration, a is the concentration of each of the hydrolytic products, m_0 is the slope of the line relating optical density to the concentration of the substrate. Similar slopes for each of the two hydrolytic products are represented by m_1 and m_2 , and D_x is the optical density of any sample drawn from the hydrolysis mixture.

From equation (1) the following relationship is readily derived:

$$\% \text{ hydrolysis} = k(D_x - D_0) \quad (2)$$

with k equal to $100/(a_0(m_1 + m_2 - m_0))$ and D_0 representing the optical density yielded by the initial substrate concentration. If unknown sam-

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† Research Fellow of the National Institutes of Health, United States Public Health Service.

ples are read in the photometer against D_0 as a "blank," equation (2) reduces to

$$\% \text{ hydrolysis} = k \cdot D_s \quad (3)$$

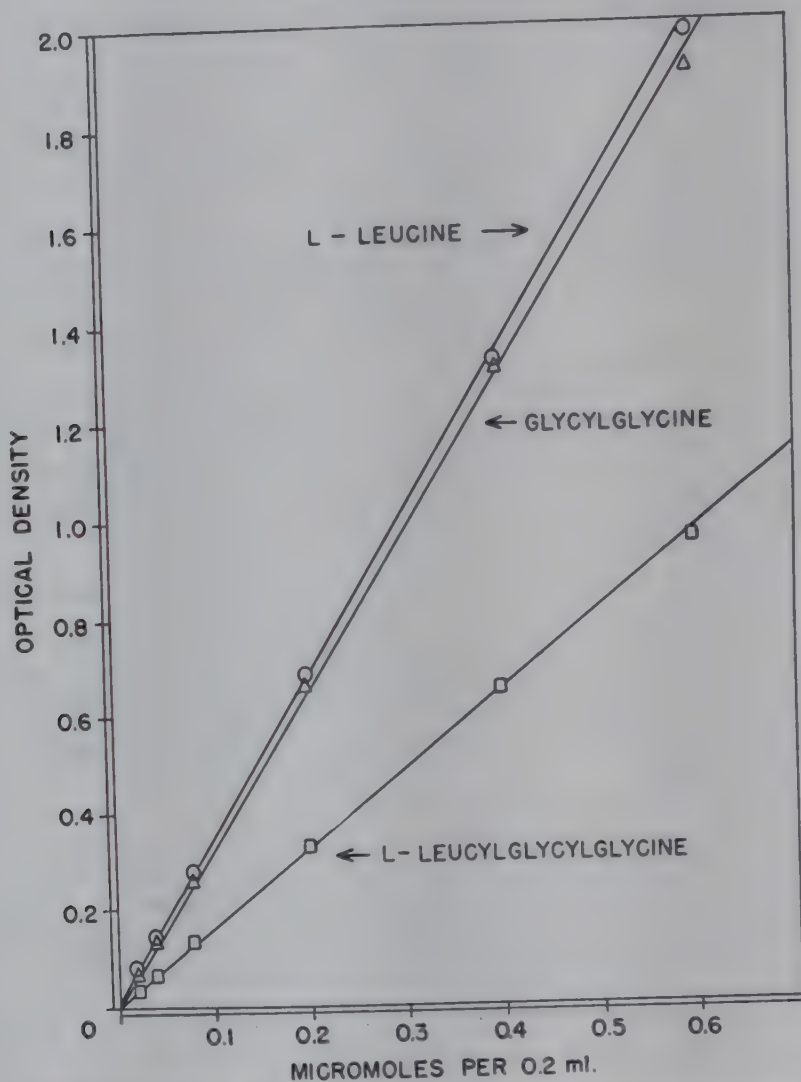


FIG. 1. Curves relating the optical density of the colored complex formed with ninhydrin and the concentrations of L-leucine ($\circ$), glycylglycine ($\triangle$), and L-leucylglycylglycine ($\square$). Each point is an average of triplicate determinations.

Therefore, it may be predicted that, if mixtures of a substrate and its two hydrolytic products are prepared in proportions representing various degrees of hydrolysis, the optical densities of these mixtures, read in the photometer against the color yield of the initial substrate concentration, would be directly proportional to the degree of hydrolysis. Thus, it should be entirely feasible to use such standard mixtures to construct a standard curve from which the per cent hydrolysis of a synthetic peptide may be estimated directly.

These theoretical considerations were tested with L-leucylglycylglycine (LGG) as substrate. The hydrolytic products are assumed to be L-leucine and glycylglycine (GG) (2, 3). Fig. 1 demonstrates that, with use of the ninhydrin reaction, for LGG, GG, and L-leucine, Beer's law is followed, the slopes calculated for these curves being 1.64, 3.28, and 3.43,

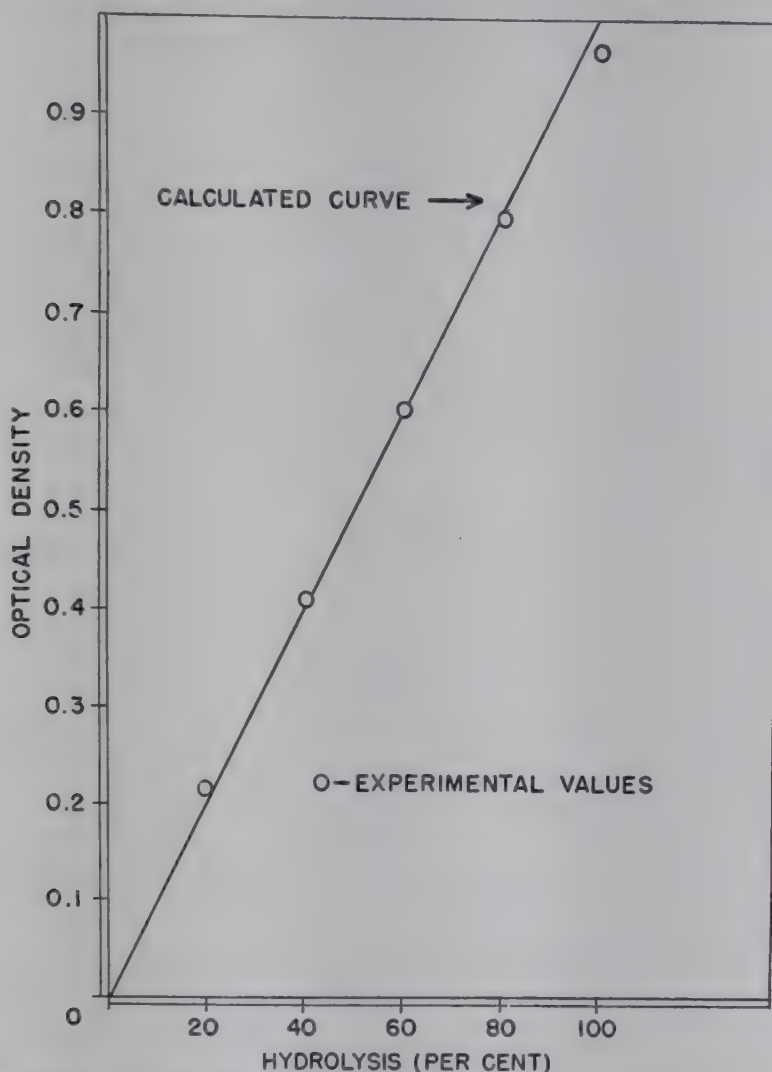


FIG. 2. Standard hydrolysis curve. The curve is plotted from equation (4) of the text. Superimposed are the values obtained from synthetic hydrolysis mixtures.

respectively. By use of an initial substrate concentration of $0.2 \mu\text{M}$ of LGG per 0.2 ml. and the above values for the slopes, k may be calculated and substituted in equation (3) as follows:

$$\% \text{ hydrolysis} = 98.8 D_x \quad (4)$$

The curve representing this relationship is plotted in Fig. 2. Its validity is substantiated by the fact that samples of solutions of LGG, GG, and

L-leucine, prepared in concentrations which would be obtained at various stages of hydrolysis when read in the photometer against D_0 , give optical densities which closely approximate the calculated hydrolysis curve. These values are plotted in Fig. 2 as well.

Materials and Methods

Reagents were prepared and used essentially as recommended by Moore and Stein (1). The ninhydrin reagent was freshly prepared immediately prior to use and yielded consistent results for only 24 hours after preparation. Enzymic hydrolysis was carried out as described elsewhere (4). Samples from hydrolysis mixtures were diluted with 1 per cent picric acid (5). The use of this reagent is advantageous in that it immediately inactivates the enzyme. Also the protein of the enzyme preparations is precipitated without affecting the substrate or its hydrolytic products, and therefore an appreciable "blank" value is not introduced. Finally, it has a very low optical density at the wave-length at which samples are read in the photometer.

Procedure

Since the values for all samples are a function of the value for the 0 minute hydrolysis, *i.e.* D_0 of equation (2), it is important that the latter value be determined for each hydrolysis mixture. This is accomplished as follows: The required volume of the enzyme preparation is added to the buffered substrate mixture which has been cooled to 4°. After shaking the resultant hydrolysis mixture for 1 minute, a sample is withdrawn and inactivated as described below. The flask containing the hydrolysis mixture is then removed from its ice bath and quickly placed in the Warburg bath¹ at 37°. Each sample (including the 0 minute hydrolysis) withdrawn from the hydrolysis flask is added to a volumetric flask which has been previously partially filled with 1 per cent picric acid. Concentrations are chosen so that, when later filled to the mark with picric acid, the volumetric flask contains 1 μM per ml. of substrate mixture. Thus in the enzymic hydrolysis reported below, the concentration of the substrate in the hydrolysis mixture was 25 μM per ml., and each 0.2 ml. sample was diluted in a 5 ml. volumetric flask to give a final concentration of 1 μM per ml. of substrate.

After all the required samples have been collected, inactivated, and

¹ It should be noted that this method of obtaining the 0 minute hydrolysis sample introduces an error. The enzymic reaction is timed from the moment the flask is placed in the Warburg bath, but we have found that approximately 7 minutes are required for the temperature of the hydrolysis mixture to rise from its initial value of 4° to 37°, the temperature of the bath. However, the temperature rises to 35° asymptotically, causing an increase to 35° in 2 minutes, and this source of error has been ignored.

appropriately diluted, they are transferred to centrifuge tubes and, after centrifugation at moderate speed for 15 minutes, the clear supernatants are decanted and, if desired, stored in a refrigerator until photometric analysis is undertaken.

Triplicate 0.2 ml. aliquots, containing 0.2 μM of substrate, are transferred to clean, matched photometer tubes. Water blanks, a standard 0 per cent hydrolysis mixture (prepared from LGG), and an arbitrarily

TABLE I

Hydrolysis of L-Leucylglycylglycine by Normal Rat Serum

The hydrolysis mixture contained 0.02 M veronal (pH 8.0), 0.05 mM of LGG, 0.2 ml. of serum, and 0.85 per cent NaCl to bring the final volume to 2 ml. Hydrolysis performed at 36.8°. Determinations I, II, and III are on the same samples on 3 successive days. The optical density was measured in the Coleman junior, model 6A, spectrophotometer.

Sample	Determination I			Determination II			Determination III		
	Optical density	Per cent hydrolysis	K ^o LGG	Optical density	Per cent hydrolysis	K ^o LGG	Optical density	Per cent hydrolysis	K ^o LGG
Blank	0.059*			0.084			0.059		
Standard 0% hydrolysis	0.320†			0.317			0.327		
Standard 40% hydrolysis	0.400‡			0.406			0.396		
0 min.	0.439§			0.430			0.472		
30 "	0.128§	12.8	0.43	0.129	12.7	0.42	0.141	14.2	0.47
60 "	0.246§	24.6	0.41	0.258	25.4	0.42	0.250	25.2	0.42
90 "	0.336§	33.6	0.37	0.350	34.5	0.38	0.327	33.0	0.37
120 "	0.435§	43.5	0.36	0.456	45.0	0.37	0.455	45.9	0.38
24 hr.	1.02§	102.0		1.06	104.0		1.00	100.9	

* Read against propanol water as a blank.

† Read against blank sample.

‡ Read against standard 0 per cent hydrolysis as a blank.

§ Read against 0 minute sample as a blank.

chosen standard 40 per cent hydrolysis mixture (prepared from LGG, LGG, and L-leucine) are similarly prepared in triplicate. From this point the procedure is essentially that recommended in the detailed paper of Moore and Stein (1). 1 ml. of ninhydrin reagent is added to each tube which then is stoppered immediately. The ninhydrin reagent is extremely sensitive, yielding falsely high optical density values if, for instance, tobacco smoke is present in the laboratory air. The photometer tubes are briefly shaken and the stoppers removed; then the tubes are immersed in a steam bath for 20 minutes, cooled in tap water, and each tube then diluted with 10 ml. of propanol-water (1).

The photometer tubes are read in a Coleman junior, model 6A, spectrophotometer² at a wave-length of 570 m μ . The tubes containing standard 0 per cent hydrolysis mixture and 0 minute hydrolysis mixture are read against the water blank tube most closely approximating the average of the three water blanks. Then, the tubes containing the standard 40 per cent hydrolysis mixture are read against the tube of the "average" standard 0 per cent hydrolysis mixture, and, finally, the remainder of the hydrolysis mixture tubes is read against the "average" 0 minute hydrolysis mixture tube. The optical densities of triplicate samples usually check within 0.01 and rarely show variations as great as 0.02. Per cent hydrolysis is calculated by multiplying the average optical density of each triplicate sample by the constant, k , which is obtained by dividing 40 by the average optical density of the 40 per cent hydrolysis standard.

Results

As representative examples of the application of this procedure, Table I shows the results obtained in estimating the rate of hydrolysis of LGG by rat serum. Three determinations on successive days were carried out on variously timed samples from a single enzymic hydrolysis. Note that the three determinations check fairly closely.

K^0_{LGG} (the per cent hydrolysis per minute for 0.1 ml. of serum per ml. of hydrolysis mixture) remains fairly constant and is comparable to values previously determined (4) for normal rat serum by the manometric method of Van Slyke and associates (5). A further check is furnished by the fact that, at 24 hours, when hydrolysis may be assumed to be complete, values approximating 100 per cent hydrolysis are obtained.

SUMMARY

A simple, accurate method, based on the ninhydrin procedure of Moore and Stein, for the photometric determination of the degree of enzymic hydrolysis of synthetic peptide substrates is described.

The helpful suggestions of Dr. Stanford Moore and the guidance of Dr. Hans Neurath are gratefully acknowledged.

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² We have also employed a Lumetron colorimeter with a 575 m μ monochromatic filter with comparable results.

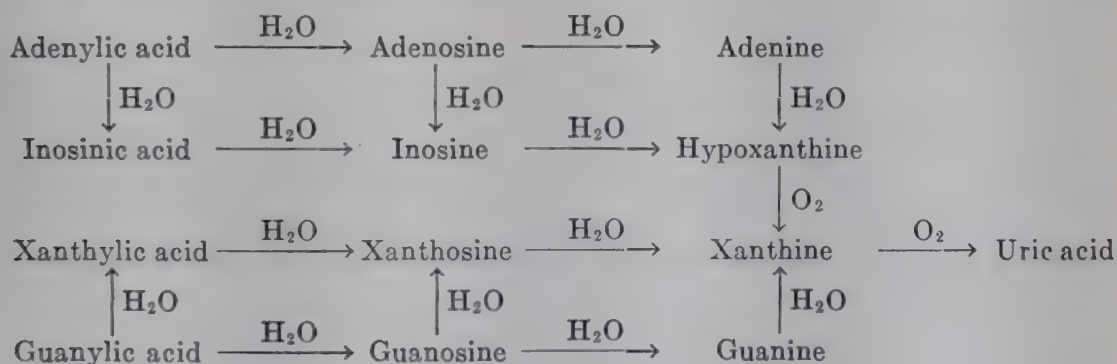
PURINE METABOLISM IN RAT LIVER HOMOGENATES*

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The idea that uric acid formation results from the graded action of several specific enzymes upon purine compounds was conceived in the laboratories of Jones and of Levene. The former (1) postulated that once nucleic acids were degraded to nucleosides guanosine led to the formation of uric acid through xanthosine and xanthine, and the adenine of adenosine was converted to uric acid by the formation first of inosine, then of hypoxanthine, and finally of xanthine. An alternative pathway was the liberation of free adenine and guanine, which could then be deaminated by adenase and guanase respectively if these enzymes happened to be present in the tissue under consideration.



The diagram shows the possible metabolic pathways for the nucleotides, nucleosides, and free purines in so far as the enzymes involved are known to exist (1-12). The only oxidative steps are those in which free hypoxanthine and xanthine are converted to uric acid and these are the only substrates in this scheme that are oxidized by xanthine oxidase. A failure to demonstrate the existence of enzymes capable of directly oxidizing nucleosides or nucleotides does not preclude their existence, and it is significant that uric acid riboside was isolated from beef erythrocytes by Davis, Newton, and Benedict (13). Our attention was drawn to the possible existence of an enzyme system capable of by-passing xanthine oxidase in the formation of uric acid because rat liver can be rendered essentially free of xanthine oxidase by dietary means (14, 15) without affecting the uric acid or allantoin excretion of the rat (unpublished).

* Aided by a grant from the Nutrition Foundation, Inc.

The possibility of practically eliminating xanthine oxidase from the liver of an intact rat by feeding a purified low protein diet presented an opportunity for determining the importance of this enzyme in purine oxidation. If the oxidative steps in purine metabolism were limited to the oxidation of xanthine and hypoxanthine, a liver homogenate free of xanthine oxidase would show no increased oxygen uptake upon the addition of any of the nucleosides and nucleotides. It was found that homogenates prepared from such rat livers of zero xanthine oxidase activity were likewise incapable of oxidizing other purines, nucleosides, or nucleotides. Restoration of this enzyme in the homogenate by the addition, *in vitro*, of purified xanthine oxidase from milk also restored the capacity of the homogenate to oxidize the other substrates. It can be concluded that in rat liver xanthine oxidase is the only oxidizing enzyme available for purine substrates, and that no evidence could be obtained for the presence of any enzyme system capable of by-passing xanthine oxidase in the formation of uric acid. It may also be noted that the other enzymes involved in the metabolism of purines, purine nucleosides, and nucleotides are not eliminated from the liver by the dietary procedure responsible for depleting xanthine oxidase.

EXPERIMENTAL

Liver xanthine oxidase was determined by the method of Axelrod and Elvehjem (16, 15), by the use of 0.15 cc. of 0.05 M xanthine in 0.05 N NaOH as the substrate. All other substrates, except guanine, were studied simultaneously by substituting 0.15 cc. of a 0.05 M solution of substrate in 0.05 M NaOH for the xanthine in an otherwise identical set-up. 0.3 cc. of a saturated solution of guanine in 0.025 N NaOH (0.0109 M by nitrogen determination) was used. All the nucleosides and nucleotides studied were D-ribose derivatives.¹

With all of the substrates, as in the xanthine oxidase determination, the endogenous respiration of the liver homogenate was allowed to proceed for 40 minutes before the substrates were tipped in. The xanthine oxidase activity of the liver in units (c.mm. of O₂ per gm. of dry liver per hour) was calculated from the net excess oxygen consumption in the flasks containing xanthine during the period when the rate was maximum and linear. Similarly, the maximum excess oxygen consumption determined with the other substrates during any 20 minute reading period was chosen for comparison of the relative rates of metabolism. With normal liver

¹ The adenosine-5-phosphoric acid was obtained from the Ernst Bischoff Company, Inc., ATP from the Nutritional Biochemicals Corporation and all other substrates from the Schwarz Laboratories, Inc. The substrates were used without further purification.

all of the substrates except adenine, guanylic acid, and adenosine-3-phosphate yielded a total net oxygen consumption approaching the theoretical values. Adenine was not oxidized at all, while the 3-phosphate derivatives of guanosine and adenosine gave 20 to 30 per cent of theory in the usual 100 minute period.

The oxygen consumption rates for the various substrates were studied with rat liver homogenates whose xanthine oxidase activities were (1) normal, (2) low, (3) zero, and (4) zero, but to which purified milk xan-

TABLE I

Net Oxygen Consumption Due to Addition of Purines and Related Substances to Rat Liver Homogenates of Varying Xanthine Oxidase Activities

Substrate	Maximum O ₂ consumption per 20 min. period when xanthine oxidase activities averaged			
	1710 units	630 units	0 units	1340 units*
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
Xanthine.....	48.5	18	0	38
Hypoxanthine.....	40	14	0	33
Adenine.....	0	0	0	
Guanine.....	45	28	0	32.5
Guanosine.....	58	32	0	56
Guanosine-3-phosphate.....	22	23	2†	28.5
Xanthosine.....	46	25	0	47
Adenosine.....	59.5	20	0	50
Adenosine-5-phosphate.....	56	22	0	42
Adenosine-3-phosphate.....	10	16	0	13

* Liver homogenate with zero xanthine oxidase activity, to which purified milk xanthine oxidase was added *in vitro*; the milk enzyme alone gave an oxygen uptake of 27 c.mm. per 20 minutes with xanthine as substrate.

† After further purification of guanylic acid by silver nitrate (27), a zero value was found.

thine oxidase was added. Normal xanthine oxidase levels were obtained in adult rats maintained on Purina dog chow. Liver xanthine oxidase was depleted to the low and zero levels by feeding adult rats a purified 8 per cent casein diet for 7 to 8 weeks (17). The milk enzyme was prepared by the method of Ball (18) and did not oxidize any of the substrates except xanthine and hypoxanthine.

Results

Table I shows the maximum rate of oxygen consumption for the various substrates when they were added to livers whose xanthine oxidase activities were 1710, 630, 0, and 0 restored to 1340 units by the addition of purified milk xanthine oxidase *in vitro*. All figures are the averages obtained in three or more experiments.

Normal rat liver homogenate (1710 units of xanthine oxidase) was capable of oxidizing all of the substrates tested except adenine; the 3-phosphate nucleotides were oxidized at a much slower rate than the other compounds, indicating that hydrolytic removal of the 3-phosphate group was relatively slow (*e.g.*, compare adenosine-5-phosphate with adenosine-3-phosphate). The oxygen consumption resulting from the addition of all these substrates was actually due to the oxidation of hypoxanthine and xanthine liberated from them by hydrolytic enzymes. Since the rates of oxygen consumption with the nucleosides and adenosine-5-phosphate exceeded the rates with xanthine and hypoxanthine, it can be concluded that the hydrolytic steps were more rapid than the oxidative steps and that the limiting enzyme in the rate of formation of uric acid was xanthine oxidase. The rate of oxidation of the hypoxanthine and xanthine formed *in situ* from guanosine and adenosine was more rapid than the rate obtained with added xanthine itself. This seems to be another manifestation of the inhibiting effect of an excess of substrate on xanthine oxidase (15); when the xanthine in the reaction vessel was formed from a nucleoside at the same time that it was being oxidized, no great accumulation of xanthine occurred, and a maximum rate of oxygen consumption was observed. The results also emphasize the danger of drawing conclusions from reaction rates obtained with complex systems, for all of the widely varying rates of oxygen uptake noted in Table I were due to the same enzyme, xanthine oxidase, acting on the same substrates, xanthine and hypoxanthine.

As the xanthine oxidase was removed from the liver by the feeding of a low protein diet, liver homogenates exhibited a decreased ability to oxidize any of the substrates except the 3-phosphate nucleotides. The decrease was relatively greater with the hypoxanthine-yielding substrates (adenosine and adenosine-5-phosphate) than with the xanthine-yielding substrates (guanine, guanosine, and xanthosine). When the liver homogenate was inactive toward xanthine, it was also inactive toward all the other substrates. The addition of purified xanthine oxidase, whose activity toward these substrates was limited to xanthine and hypoxanthine, restored the capacity of the homogenate to oxidize all of the substrates. This demonstrated the presence in the homogenate of all the enzymes required to convert the nucleosides and nucleotides to xanthine and hypoxanthine, and showed that the failure to oxidize such substrates was due to a deficiency of xanthine oxidase only. The rate of oxidation of each substrate was restored proportionately to about the degree found in normal liver, indicating that no unidentified nucleoside or nucleotide oxidative enzymes were present in normal liver which were also removed concomitantly with the xanthine oxidase by feeding a low protein diet. It cannot

be inferred that the non-oxidative enzymes involved in nucleotide and nucleoside metabolism were completely unaffected by the low protein diet, but it can be concluded that they were not affected sufficiently to make them limiting factors in the formation of uric acid.

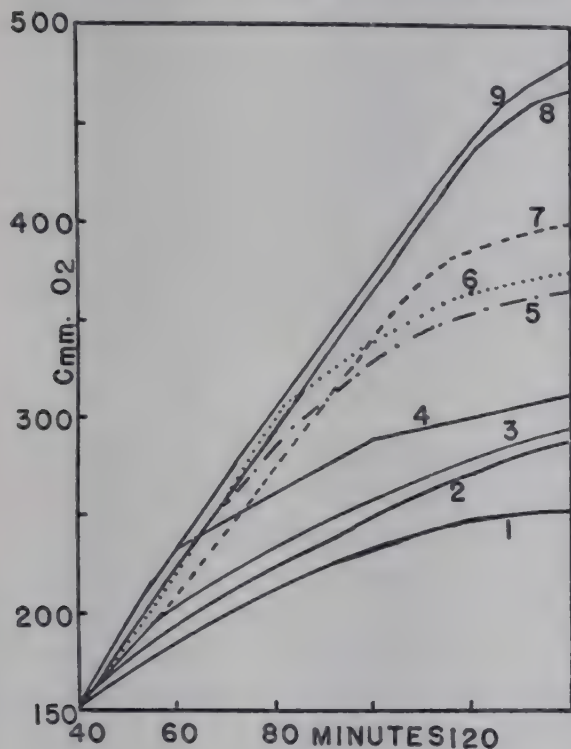


FIG. 1

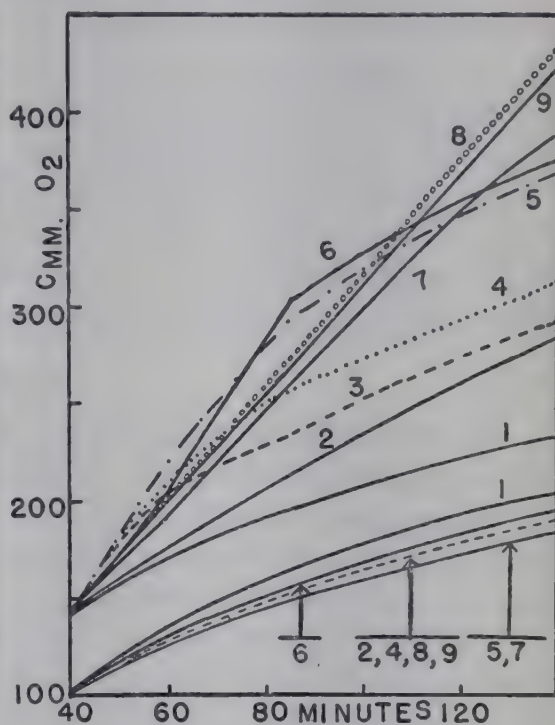


FIG. 2

FIG. 1. Oxygen consumption curves for purine substrates added to a normal liver homogenate. Each substrate was tipped in after the liver homogenate had respired for 40 minutes. Curve 1, continued endogenous respiration of the liver; Curve 2, adenosine-3-phosphate; Curve 3, guanosine-3-phosphate; Curve 4, guanine; Curve 5, xanthosine; Curve 6, guanosine; Curve 7, xanthine; Curve 8, adenosine; and Curve 9, adenosine-5-phosphate.

FIG. 2. Oxygen consumption curves for purine substrates added to a liver of zero xanthine oxidase activity in the presence and absence of added purified milk xanthine oxidase. Lower set of curves, liver homogenate of zero xanthine activity into which the substrates were tipped after 40 minutes; curve numbers as in Fig. 1. Upper set of curves, same liver homogenate to which purified milk xanthine oxidase was added *in vitro* to the main body of the Warburg flask at the start; substrates were tipped in after 40 minutes; curve numbers as in Fig. 1.

Fig. 1 shows the oxygen consumption curves for the various substrates added to a normal liver homogenate (xanthine oxidase, 1470 units). The curves in Fig. 2 were similarly obtained when the substrates were added to a liver of zero xanthine oxidase activity, with and without the addition of purified milk xanthine oxidase (30 c.mm. of O_2 per 20 minutes).

Adenosine triphosphate (ATP) was metabolized by a liver homogenate,

whose xanthine oxidase activity was 2170 units, at a rate of 45 c.mm. of O_2 per 20 minutes. Schmidt and Thannhauser (19) showed that the alkaline phosphatase from calf small intestine could hydrolyze all three phosphate linkages of ATP, the two pyrophosphate bonds being split first. If this were also true for rat liver homogenates, ATP would be converted to adenylic acid and then follow the subsequent metabolic pathway of this compound. The excess oxygen consumption resulting from the addition of 0.025 M yeast nucleic acid or desoxyribonucleic acid to a normal liver homogenate was low, 9 c.mm. of O_2 per 20 minutes, indicating that either (1) the hydrolysis of the nucleic acid to its constituent nucleotides was a relatively slow process in comparison to the subsequent breakdown of the nucleotides, or (2) the nucleotides derived from nucleic acid in rat liver homogenates were the 3-phosphate derivatives.

DISCUSSION

The formation of hypoxanthine from adenylic acid and adenosine in the absence of adenase from rat liver places inosine as the key intermediate in this process. Whether adenylic acid was dephosphorylated to adenosine in preference to being deaminated to inosinic acid prior to the formation of inosine could not be determined from these studies. Available evidence favors adenosine as the major but not necessarily the exclusive breakdown product of adenylic acid in liver (20, 21) and inosinic acid as the principal intermediate in skeletal muscle (20, 22-24). There seem to be no other possible alternatives in the case of rat liver, since none of the nucleotides or nucleosides were capable of being oxidized directly. The data fail to indicate a preferential pathway for the metabolism of guanylic acid, and all of the theoretical pathways previously indicated remain possibilities in rat liver.

The synthetic utilization of dietary adenine by the rat, in contrast to guanine (25), may reflect the relative absence of adenase and the presence of guanase in rat tissues, and thus the fed adenine remains available for synthetic reactions while the guanine is rapidly converted to allantoin and excreted. Rat tissues are not completely free of adenase, some of the enzyme being found in the intestinal mucosa (26), but adenase appears to be distributed very sparsely in animal tissues in comparison with guanase.

SUMMARY

Normal rat liver homogenate gave an increased oxygen uptake upon the addition of guanine, guanosine, xanthosine, adenosine, and adenosine-5-phosphate, owing to the oxidation of xanthine and hypoxanthine formed from these substrates by enzymatic hydrolytic reactions. The 3-phos-

phate derivatives of guanosine and adenosine were hydrolyzed slowly. Adenine gave no oxygen uptake owing to the absence of adenase from rat liver.

When the xanthine oxidase was removed from the liver by feeding a purified low protein diet, the homogenate was incapable of oxidizing any of the above substrates. The addition of purified xanthine oxidase from milk to such a liver preparation *in vitro* restored its normal capacity to oxidize all of the substrates. Hence, no unidentified nucleoside or nucleotide oxidative enzymes were present in rat liver, and none of the hydrolytic enzymes involved in nucleotide metabolism were decreased by feeding a low protein diet to the point where they became limiting factors in the rate of uric acid formation.

It was concluded that inosine is a key intermediate in the formation of uric acid from adenylic acid in rat liver.

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OBSERVATIONS ON THE USE OF LACTOBACILLUS LEICHMANNII 4797 IN THE MICROBIOLOGICAL ASSAY OF VITAMIN B₁₂

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Lactobacillus leichmannii (ATCC 4797) was reported by Skeggs *et al.* (1) to grow in an otherwise complete medium in response to increasing amounts of the "animal protein factor." The reports of Ott *et al.* (2) and Emerson (3) have demonstrated the probable identity of crystalline vitamin B₁₂ with the "animal protein factor." The use of another strain of *L. leichmannii* (313) in the microbiological assay for vitamin B₁₂ has been reported by Hoffmann *et al.* (4), and subsequent investigations have shown that strain 4797 of *L. leichmannii* also responds to crystalline vitamin B₁₂. In the course of our investigations of crystalline vitamin B₁₂ in the nutrition of *L. leichmannii*, the various modifications in the basal medium and testing conditions that are described in this paper have been developed.

EXPERIMENTAL

The usual procedures for microbiological assay have been employed. The assays have been carried out in a final volume of 10 ml. in 20 × 150 mm. test-tubes.

Crystalline vitamin B₁₂ (Merck) has been used as a standard over a range of 0.025 to 0.25 mμgm. per tube.

L. leichmannii (4797) was carried in stock culture by monthly transfer in skim milk (Difco) supplemented with 1 per cent tryptose (Difco). For the preparation of the inoculum the organism also was carried by daily transfer in this medium. It has been found advisable to return to stock at 1 or 2 week intervals.

The inoculum was prepared by suspending 0.1 ml. of a 24 hour milk culture in approximately 10 ml. of sterile physiological saline, from which a second dilution of 1:10 was prepared for seed. One drop of the second dilution was added aseptically to each assay tube following sterilization of the test. Sterilization was accomplished by autoclaving the cotton-plugged assay tubes (containing test solution or standard in appropriate amounts, 5 ml. of basal medium, and sufficient water to make a volume of 10 ml.) for 15 minutes at 120°.

Assay results could be obtained turbidimetrically after 24 hours incubation at 37° or by titration with 0.1 N NaOH after 72 hours incubation with comparable results.

The basal medium is shown in Table I.

Casein Hydrolysate—The method of preparation of the casein hydrolysate is of utmost importance. Repeated adsorption of the acid-hydrolyzed casein on carbon results in a poor response of the organism to vitamin B₁₂ and increases the requirement of *L. leichmannii* for that growth factor. The casein hydrolysate is prepared as follows: 100 gm. of Labco vitamin-free casein are refluxed for 8 to 10 hours with 500 ml. of concentrated HCl and 500 ml. of H₂O. The HCl is distilled off under a vacuum, the

TABLE I
*Composition of Double Strength Medium**

Casein hydrolysate*	1.0 gm.	Pyridoxal	400 γ
Glucose	4.0 "	Riboflavin	200 "
Tryptophan	20 mg.	Nicotinic acid	200 "
Cystine	20 "	Pantothenic acid	200 "
Adenine	1 "	Thiamine	200 "
Guanine	1 "	Folic acid	100 "
Xanthine	1 "	<i>p</i> -Aminobenzoic acid	100 "
Uracil	1 "	Tween 80	0.2 ml.
Salts A†	1 ml.	Thiomalic acid (recrystallized)*	100 mg.
" B†	1 "		
Na acetate (anhydrous)	1.2 gm.	Ribonucleic, guanylic, or uridylic acid*	25 "
Biotin	1 γ		
Pyridoxine	400 "	Distilled water to 100 ml.	

* See the text.

† Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).

volume is restored with H₂O, and the evaporation *in vacuo* is repeated. The hydrolyzed casein is dissolved in approximately 800 ml. of H₂O and is adjusted to pH 3.0 with 10 per cent NaOH. It then is filtered and the filtrate is stirred for half an hour at room temperature with 10 gm. of Darco G-60. Following filtration the hydrolysate is combined with the sodium acetate, purines, and pyrimidines, Salts A and B, tryptophan, and cystine. The mixture is adjusted to pH 6.6 to 6.8, diluted to 10 liters with H₂O, and stored under benzene. The remaining components of the medium are added proportionately to an appropriate aliquot just prior to use in an assay.

In early studies with the animal protein factor, trypsinized casein had been included in the basal medium (1) as a source of streptogenin. When crystalline vitamin B₁₂ became available for study, it was found that the

trypsinized casein could be omitted, since it had no demonstrable effect on the response of the organism to the crystalline vitamin.

Thiomalic Acid—Preliminary work on the heat stability of vitamin B₁₂ indicated that the vitamin in crude preparations was not stable. Stability curves obtained with crude liver preparation showed a progressive loss of activity as the autoclaving time increased, which leveled off at 20 to 30 per cent, but never reached zero. As shown in Fig. 1, the crystalline vitamin, subjected to the same autoclaving conditions, was relatively stable.

Following the report of Stokstad *et al.* (5) that thioglycolic acid and various reducing substances protected the vitamin B₁₂ in crude materials from heat destruction during autoclaving, a survey was made to determine

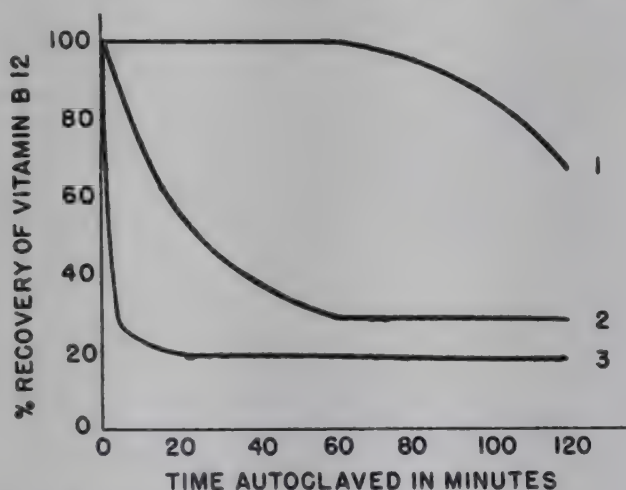


FIG. 1. Stability of vitamin B₁₂ preparations. Curve 1, vitamin B₁₂, Merck; Curve 2, partially purified "animal protein factor;" Curve 3, crude Viobin liver.

which antioxidant would prove most satisfactory for routine use. Thioglycolic acid, cysteine, ascorbic acid, isoascorbic acid, sodium hydro-sulfite, ethylenediaminetetraacetic acid (Versene), and thiomalic acid were equally effective in preventing the destruction of vitamin B₁₂ in crude preparations. Thiomalic acid was chosen for further study because, in addition to its consistent effectiveness over a wide range in protecting vitamin B₁₂ against destruction in crude materials, it improved the growth response of *L. leichmannii* to crystalline vitamin B₁₂. The response of *L. leichmannii* to vitamin B₁₂ in the presence of thiomalic acid or of thioglycolic acid is compared in Fig. 2. Growth with the latter compound is the same as that obtained in the absence of any reducing agent. The growth stimulation obtained with thiomalic acid apparently is due to the organic acid itself, since malic acid and related organic acids stimulate the growth of *L. leichmannii* in the presence of vitamin B₁₂, particularly

during the first 24 hours. The effects of various acids (all at a concentration of 1 mg. per tube) are shown in Fig. 3; they can be obtained either in the absence of reducing agents or in the presence of a non-stimulatory antioxidant, such as Versene.

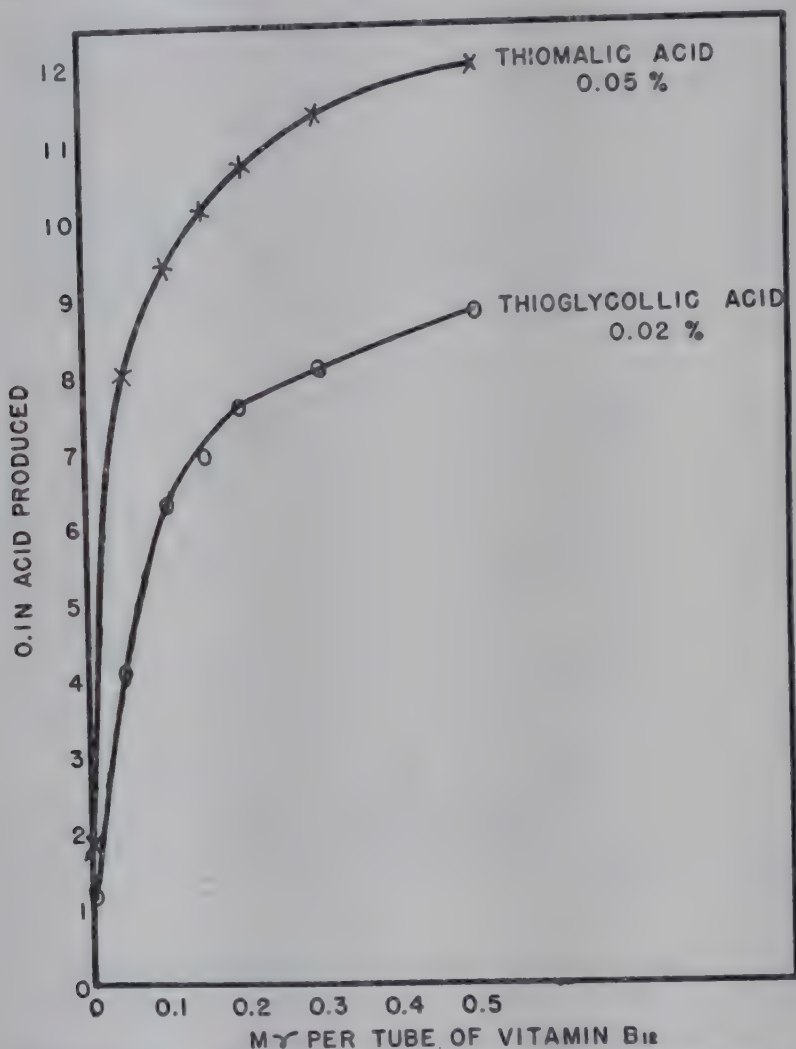


FIG. 2. Response of *L. leichmannii* 4797 to vitamin B₁₂ in presence of thiomalic acid or thioglycolic acid.

In previous studies it had been noted that failure to autoclave the glucose in the test medium, insufficient autoclaving time, or autoclaving at lower temperatures resulted in a very poor response of the organism to vitamin B₁₂. Stokstad *et al.* (5) found that the addition of reducing substances to the medium served to initiate growth in cases in which the glucose had not been autoclaved. Thiomalic acid also functions in this way and makes the time of autoclaving and temperature of sterilization factors that may be varied at will. Comparable assay results have been obtained

by autoclaving 3, 10, or 15 minutes at 120° or by steaming 15 minutes at 100°.

Welch and Wilson (6) have noted that the ability of ascorbic acid to replace vitamin B₁₂ is dependent upon the presence in the medium of a tryptic digest of casein which in the absence of the reducing agent supports no growth. Thiomalic acid functions similarly and will replace vitamin B₁₂ completely if an apparently inactive preparation of trypsinized casein is included in the medium. One explanation may be that, since the reducing agents serve to protect B₁₂ in crude material against heat destruc-

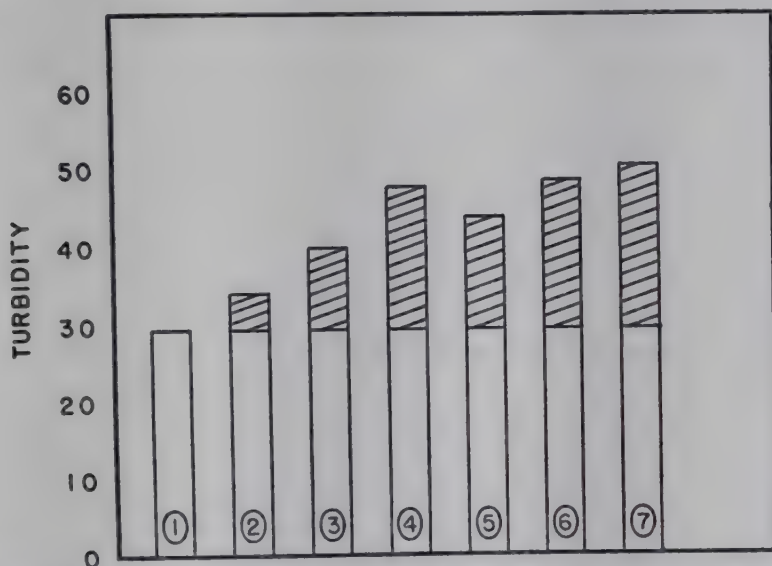


FIG. 3. Effect of organic acids (1 mg. per tube) on 24 hour growth response of *L. leichmannii* (4797) to vitamin B₁₂ (0.0005 γ per tube). 1, vitamin B₁₂ alone; 2, vitamin B₁₂ + oxalacetic acid; 3, vitamin B₁₂ + fumaric acid; 4, vitamin B₁₂ + malic acid; 5, vitamin B₁₂ + succinic acid; 6, vitamin B₁₂ + citric acid; 7, vitamin B₁₂ + α -ketoglutaric acid. The turbidity was measured in the Klett-Summerson photoelectric colorimeter.

tion, enzymatically digested casein, even after adsorption on carbon, may retain a considerable amount of heat-labile vitamin B₁₂ complex. In the basal medium described in Table I, increased amounts of thiomalic acid do not replace vitamin B₁₂ in the nutrition of *L. leichmannii*. On the other hand, when either crystalline or crude preparations of vitamin B₁₂ were autoclaved apart from the medium with either thioglycolic or thiomalic acid, their growth-promoting activity for *L. leichmannii* partially was destroyed, possibly through formation of vitamin B_{12a}. Kaczka *et al.* (7) formed this latter product by hydrogenation of vitamin B₁₂, and reported that it was less active than vitamin B₁₂ for both *L. leichmannii* and the chick. It would appear, therefore, that the action of thiomalic

acid in protecting vitamin B₁₂ against destruction in the assay procedure must result from its interaction in some unknown manner with the ingredients of the basal medium (and of the added samples) other than vitamin B₁₂. If an excess (0.5 per cent) of thiomalic acid is added to the basal medium, interaction with vitamin B₁₂ may occur, since, under these conditions, no growth response to the vitamin was obtained. Therefore the use of reducing substances in the assay procedure must be regulated carefully.

Nucleotides—The inclusion of thiomalic acid in the medium did not overcome completely the tendency of the growth response curves to flatten abruptly at a level of about 0.1 mμgm. of vitamin B₁₂; and the addition of as much as 1 mμgm. of the crystalline vitamin allowed no further growth. If liver extracts or various crude materials replaced vitamin B₁₂, growth was more rapid and a response was elicited over a wider range. Consequently the interpretation of assay results was very difficult, since the "working" range of the standard curve was limited. In an attempt to increase the span of the growth curve, various supplements to the medium were tested. Acid-hydrolyzed yeast extract or yeast ribonucleic acid, neither of which had any vitamin B₁₂ activity *per se*, promoted increased growth of *L. leichmannii* in the presence of maximal amounts of vitamin B₁₂. Ribonucleic acid (RNA), purified¹ and degraded by alkaline hydrolysis, retained this ability to promote additional growth of the organism. A study of the growth-promoting effects of the various component nucleotides and nucleosides then was undertaken. Additional purines and pyrimidines and the nucleosides were ineffective. Guanylic acid and uridylic acid stimulated further growth in the presence of vitamin B₁₂. Cytidylic acid preparations varied in that they either stimulated growth or had no effect on the response of *L. leichmannii* to vitamin B₁₂. All preparations of adenylic acid tested inhibited growth during the first 24 hours of growth, but the inhibitory effects were overcome completely on prolonged incubation. Hutchings *et al.* (8) have found that certain nucleotides are active in stimulating the growth of *Lactobacillus gayonii*. Fig. 4 shows the effect of the nucleotides and nucleic acids (1.25 mg. per tube) on the response of *L. leichmannii* to 1 mμgm. of vitamin B₁₂ in the presence of thiomalic acid at 24 and at 72 hours.

The stimulatory nucleotides are of particular value in permitting more rapid growth of the organism and a wider spacing between assay levels that lead to increased accuracy in evaluating results. This is evident by examination of the response curves of *L. leichmannii* to vitamin B₁₂

¹ We are indebted to Dr. John Spizizen of the Virus Research Department for the purified RNA used in these studies.

shown in Fig. 5. In these curves the RNA and the nucleotides were incorporated in the basal medium at a level of 1.25 mg. per tube and the curves were read turbidimetrically after 18 hours incubation at 37°. Samples assayed under similar test conditions give comparable results, however, whether or not the nucleotides and RNA are present in the medium (Table II). The inclusion of the nucleotides in the medium is determined, there-

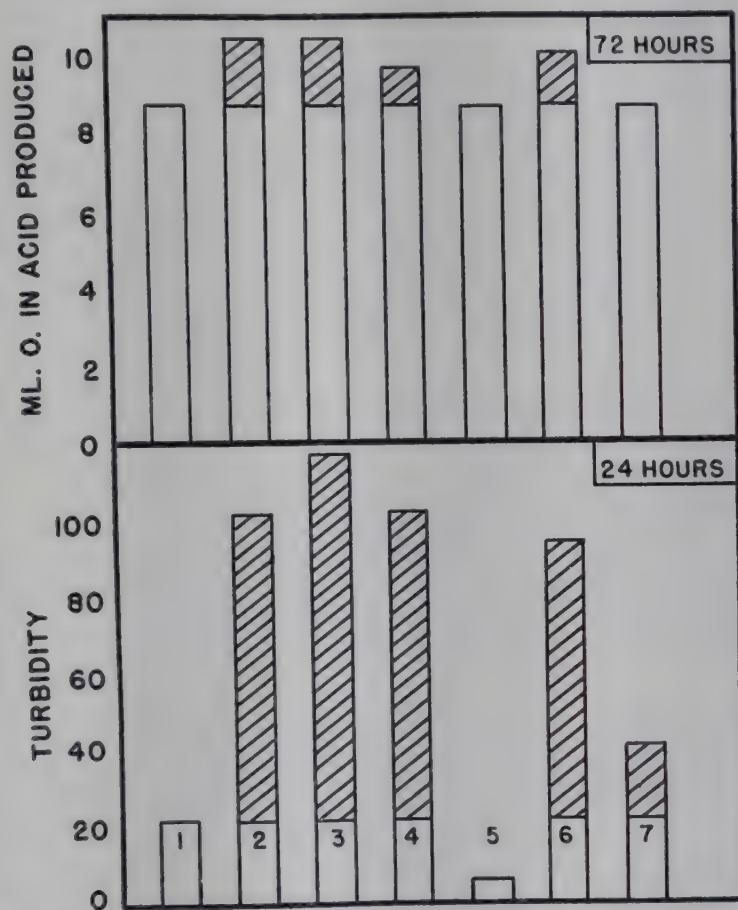


FIG. 4. Effect of nucleotides and nucleic acids (1.25 mg. per tube) on response of *L. leichmannii* (4797) to vitamin B₁₂ (0.001 γ per tube). 1, vitamin B₁₂ alone; 2, vitamin B₁₂ + guanylic acid; 3, vitamin B₁₂ + uridylic acid; 4, vitamin B₁₂ + cytidylic acid; 5, vitamin B₁₂ + adenylic acid; 6, vitamin B₁₂ + ribonucleic acid; 7, vitamin B₁₂ + desoxyribonucleic acid.

fore, by the type of sample to be assayed and the length of the incubation period to be employed. Samples of low potency that would be expected to contain relatively large amounts of nuclear material should be assayed in the presence of one or more of the stimulatory nucleotides or of RNA.

Specificity of Response—Even before *L. leichmannii* was shown to respond to crystalline vitamin B₁₂, it was found that thymidine would replace concentrates of the “animal protein factor” in the nutrition of that organism (1). Kitay *et al.* (9) subsequently proved that other desoxyribo-

sides also may function in the growth of *L. leichmannii*. Several methods of distinguishing between the growth-promoting activity of desoxyribosides and of vitamin B₁₂ may be employed.

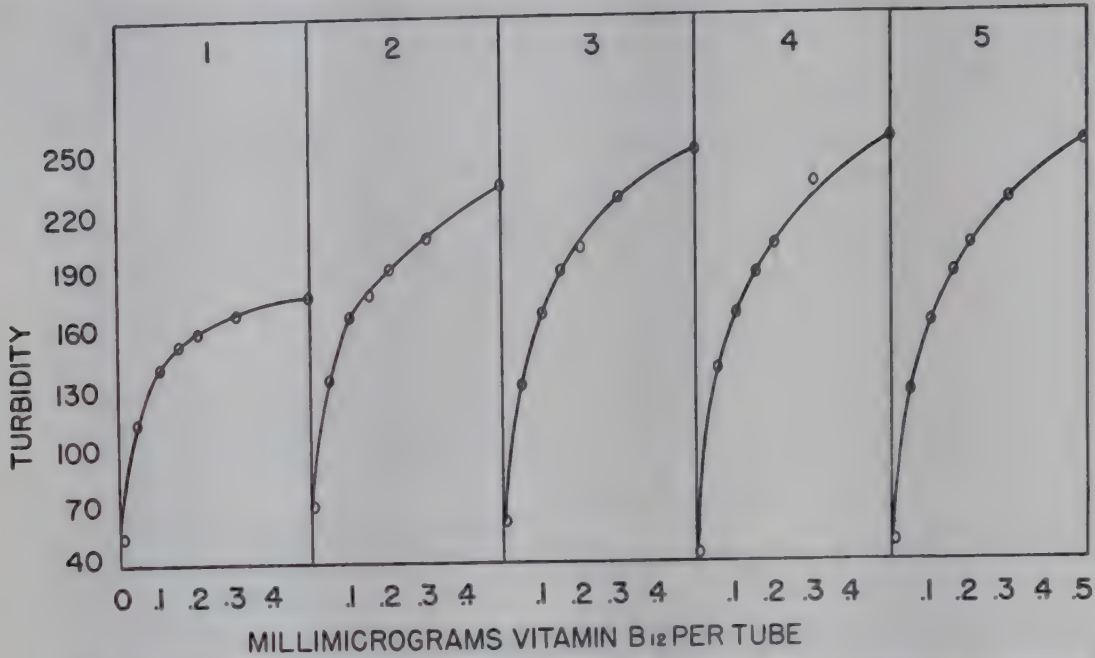


FIG. 5. Effect of nucleotides and RNA (1.25 mg. per tube) on response curves of *L. leichmannii* (4797) to vitamin B₁₂. 1, no supplement; 2, ribonucleic acid; 3, guanylic acid; 4, uridylic acid; 5, guanylic and uridylic acids.

TABLE II
Effects of Ribonucleotides on Assay for Vitamin B₁₂ in Crude Extracts

Supplement to medium		Sample 223-34F	Sample D238	Sample 4321111B
	mg. per tube	γ per ml.	γ per ml.	γ per ml.
None.....	0	0.18	0.25	1.8
Guanylic acid.....	1.25	0.17	0.22	1.7
Uridylic ".....	1.25	0.17	0.19	1.4
Guanylic ".....	1.25			
+ uridylic acid.....	1.25	0.17	0.19	1.7
Ribonucleic acid.....	1.25	0.18	0.24	1.7

In assaying for vitamin B₁₂, however, the effects of the desoxyribosides usually are eliminated simply by dilution. The activity ratio of thymidine to vitamin B₁₂, for example, is 10,000:1. The results of the separation obtained by paper strip chromatography (10) are somewhat misleading, since the ratio of activity of thymidine to vitamin B₁₂, when allowed to diffuse from paper disks on surface agar, is only 10:1. The relative inactivity of vitamin B₁₂ under such conditions probably is attributable to the

large molecular weight of the compound and its consequent slow rate of diffusion.

Recently Winsten *et al.* (11) noted the presence of a factor active in promoting the growth of *L. leichmannii* 313 in an acid precipitate of cow manure; it moved more slowly on paper strips than did either vitamin B₁₂ or the desoxyribosides. It seems possible that this factor may be desoxy-

TABLE III
Recovery of Vitamin B₁₂

Liver extract No.	Added crystalline vitamin B ₁₂	Vitamin B ₁₂ added in sample	Vitamin B ₁₂ found	Per cent recovery
	<i>mμgm. per tube</i>	<i>mμgm. per tube</i>	<i>mμgm. per tube</i>	
1	0.050	0.014	0.061	101
	0.100	0.028	0.150	
	0.150	0.042	0.200	
	0.250	0.070	0.273	
2	0.050	0.015	0.063	103
	0.100	0.030	0.132	
	0.150	0.045	0.212	
3	0.050	0.012	0.060	96
	0.100	0.025	0.127	
	0.150	0.063	0.178	
	0.250	0.038	0.295	

TABLE IV
Reproducibility of Vitamin B₁₂ Assay Procedure

Liver extract No.	Average vitamin B ₁₂ *	Range	Standard deviation
	<i>γ per ml.</i>	<i>γ per ml.</i>	
1	1.5	1.3-1.9	±0.4
2	1.7	1.5-2.1	±0.4
3	1.5	1.1-2.1	±0.6

* Average of ten determinations.

ribonucleic acid (DNA). In the regular test-tube method of assay, approximately 40 mμgm. of DNA will support partial growth of *L. leichmannii*. Increased amounts of DNA do not permit a maximal growth response of the organism. 1 mg. of DNA, added to an optimal amount of vitamin B₁₂, as shown in Fig. 4, stimulates growth during the first 24 hours but is without effect after 72 hours. Since the ratio of activity of DNA to vitamin B₁₂ in the test-tube is approximately 800,000:1, the interfering effects of DNA under ordinary assay conditions usually can be removed

by dilution. On paper strip chromatography, the two factors would be distinguishable, but the relative degree of activity of each could not be defined.

Reliability—The reliability of a given assay procedure can be assessed in terms of recovery and reproducibility. Vitamin B₁₂, when mixed with crude liver samples and subjected to the microbiological assay procedure described, can be shown to be available for the growth of *L. leichmannii*. The percentage recovery of vitamin B₁₂ is shown in Table III. Good results were not obtained in the absence of thiomalic acid.

The reproducibility of the assay procedure described is shown by the data in Table IV. The average results shown were obtained from ten assays carried out by three different individuals, with use of six separate batches of casein hydrolysate and three batches of recrystallized thiomalic acid, over a period of 2 months. Even under such varied conditions the reproducibility falls within the range usually encountered in microbiological assays. The experimental error² (after removing the effects of samples and replications by the analyses of variance) is 19.9 per cent ($P = 0.05$).

DISCUSSION

No mention has been made of the activity of vitamins B_{12a} (7) or B_{12b} (12) under the assay conditions described, since they have not been available for testing. Presumably vitamin B_{12b}, which is as active as vitamin B₁₂ for *L. leichmannii* 313, would be as active as vitamin B₁₂ for *L. leichmannii* 4797, since strain 313 has been found to behave exactly like strain 4797 in all of the procedures described, and since comparable results can be obtained with either organism. The activity of vitamin B_{12a} cannot be predicted, since its activity in an assay with *L. leichmannii* in which a reducing agent was employed has not been reported.

The microbiological assay procedure with *L. leichmannii* that has been evolved appears to constitute, on the basis of present information, a valid measure of the vitamin B₁₂ activity of a given preparation. The discrepancies between microbiological and chick assays that Stokstad *et al.* (13) have reported possibly may be explained by the presence in the particular samples of substances either that cannot be antagonized by the reducing agent present in the medium or that are present in higher amounts than those usually encountered. In the latter instance, the use of larger amounts of thioglycolic acid (or thiomalic acid) might yield microbiological data more nearly in agreement with the results obtained by chick assay.

SUMMARY

1. An assay procedure for vitamin B₁₂, employing *Lactobacillus leichmannii* 4797, has been described in detail.

² We are indebted to Mr. Joseph L. Ciminera for statistical analyses of the data.

2. The proposed procedure has been found to be reliable and reproducible.

3. Problems such as those having to do with specificity and stability that are encountered in the microbiological assay for vitamin B₁₂ have been discussed.

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THE RESPONSE OF LACTOBACILLUS CASEI TO PARTIAL HYDROLYSATES OF PROTEIN*

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It was observed in preliminary experiments that growth and acid production of *Lactobacillus arabinosus* 17-5 on a chemically defined medium (2) lacking isoleucine, leucine, or tryptophan were stimulated by partial hydrolysates of egg proteins directly in proportion to the degree of hydrolysis of the protein. *Lactobacillus fermenti* 36 (histidine, methionine, and threonine) and *Leuconostoc mesenteroides* P-60 (phenylalanine and lysine) responded similarly on the same medium lacking one of the indicated amino acids.

On the other hand the stimulatory effect of the partial hydrolysates for *Lactobacillus casei*, grown on a medium lacking arginine or tyrosine, was greatly in excess of that produced by an equivalent amount of the pure amino acid. These investigations, extended as described in the experimental part, are of particular interest because of their possible relation to streptogenin.

EXPERIMENTAL

Hydrolysis of Proteins—A mixture containing about 5 gm. of casein¹ or of crystalline bovine plasma albumin² and 50 ml. of 0.5 N HCl was refluxed for 6 hours. The hydrolysate was cooled, brought to pH 7.0 with NaOH, diluted to 250 ml. in a volumetric flask, and stored under toluene in the refrigerator. The hydrolysis was about 20 per cent complete, according to calculation by means of the formula

$$\% \text{ hydrolysis} = \frac{\% \text{ amino N in partial hydrolysate} - \% \text{ amino N in protein}}{\% \text{ amino N in complete hydrolysate} - \% \text{ amino N in protein}}$$

* Paper 62. For Paper 61, see Rockland and Dunn (1). This work has been aided by grants from Swift and Company and the National Institutes of Health, United States Public Health Service.

¹ Prepared by the method of Dunn (3). The product contained 9.55 per cent moisture, 0.63 per cent ash, and 15.35 per cent nitrogen (corrected for moisture and ash).

² The product (Lot 46), prepared by Armour and Company, Chicago, contained 7.18 per cent moisture, 0.23 per cent ash, and 17.86 per cent nitrogen (corrected for moisture and ash).

where amino nitrogen was determined by the Van Slyke manometric nitrous acid method and complete hydrolysis was achieved by treatment of the protein with 6 N HCl for 24 hours under a reflux.

Microbiological Testing Procedure—The microbiological techniques and basal medium employed were those described by Dunn *et al.* (2). The activity of the partial hydrolysates for *L. casei* was measured in terms of acid produced in the basal medium, complete except for the omission in turn of each essential amino acid. According to the criteria of Dunn *et*

TABLE I

Amino Acid Composition of Bovine Plasma Albumin and Casein

Values as per cent of ash- and moisture-free proteins.

Amino acid	Bovine plasma albumin				Casein	
	Brand (5)	Velick and Ronzoni (6)	Stein and Moore (7)	Authors	Literature*	Authors
Arginine.....	6.2	6.2	5.9	5.6	3.8	3.5
Cystine.....	6.52	5.9	5.9	6.7	0.4	0.40*
Glutamic acid†.....	16.9	17.0	16.5	22.4‡	22.0	20.9
Isoleucine.....	2.9	2.7	2.6	2.9	5.6	5.4
Leucine.....	13.7	13.2	12.3	11.4	9.3	9.1
Phenylalanine.....	6.2	6.05	6.6	6.3	4.9	4.8
Serine.....	4.5	4.9	4.2	4.5§	5.0	5.0*
Tryptophan.....	0.58	0.58		0.61	1.3	1.3*
Tyrosine†.....	5.49	5.50	5.1	4.45	5.5	5.3
Valine.....	6.5	6.6	5.9	6.0	6.7	6.6

* Cited by Dunn (8).

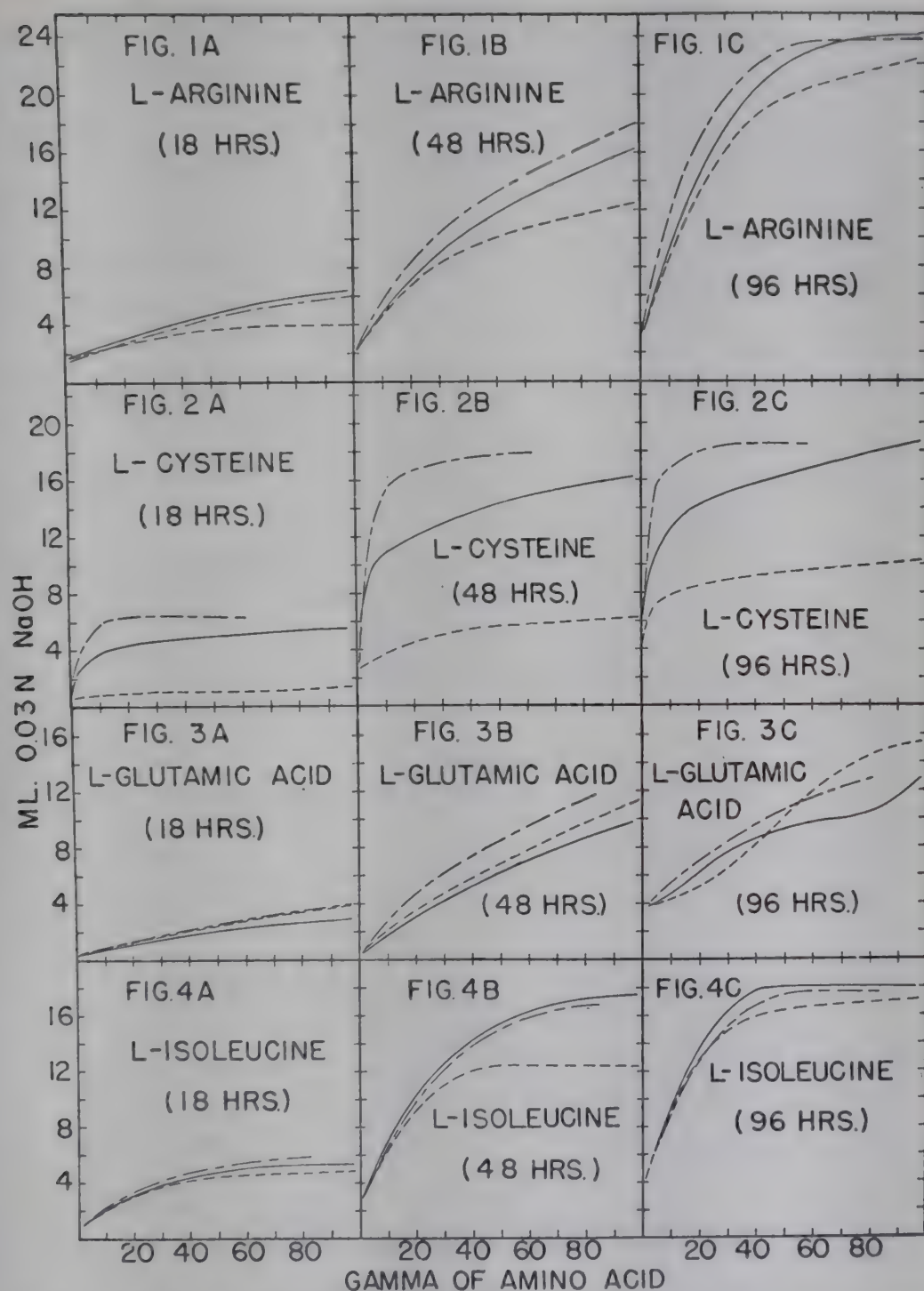
† Values, 17.0 per cent for glutamic acid and 5.53 per cent for tyrosine, found by Shemin (9).

‡ Apparently incorrect.

§ Value given by Brand (5).

al. (4), *L. casei* requires arginine, cystine, glutamic acid, isoleucine, leucine, phenylalanine, serine, tryptophan, tyrosine, and valine.³ The activity of the partial hydrolysate and of each essential amino acid was determined simultaneously in duplicate at eleven levels, ranging from 0 to 100 γ per tube. With tryptophan, the range was 0 to 50 γ per tube. The partial hydrolysates were tested at ten levels in duplicate in quantities such that the total amount of the test amino acid (free and bound) provided was equivalent to the free (pure) amino acid added to the control tubes. The concentration of the amino acid in the partial hydrolysate

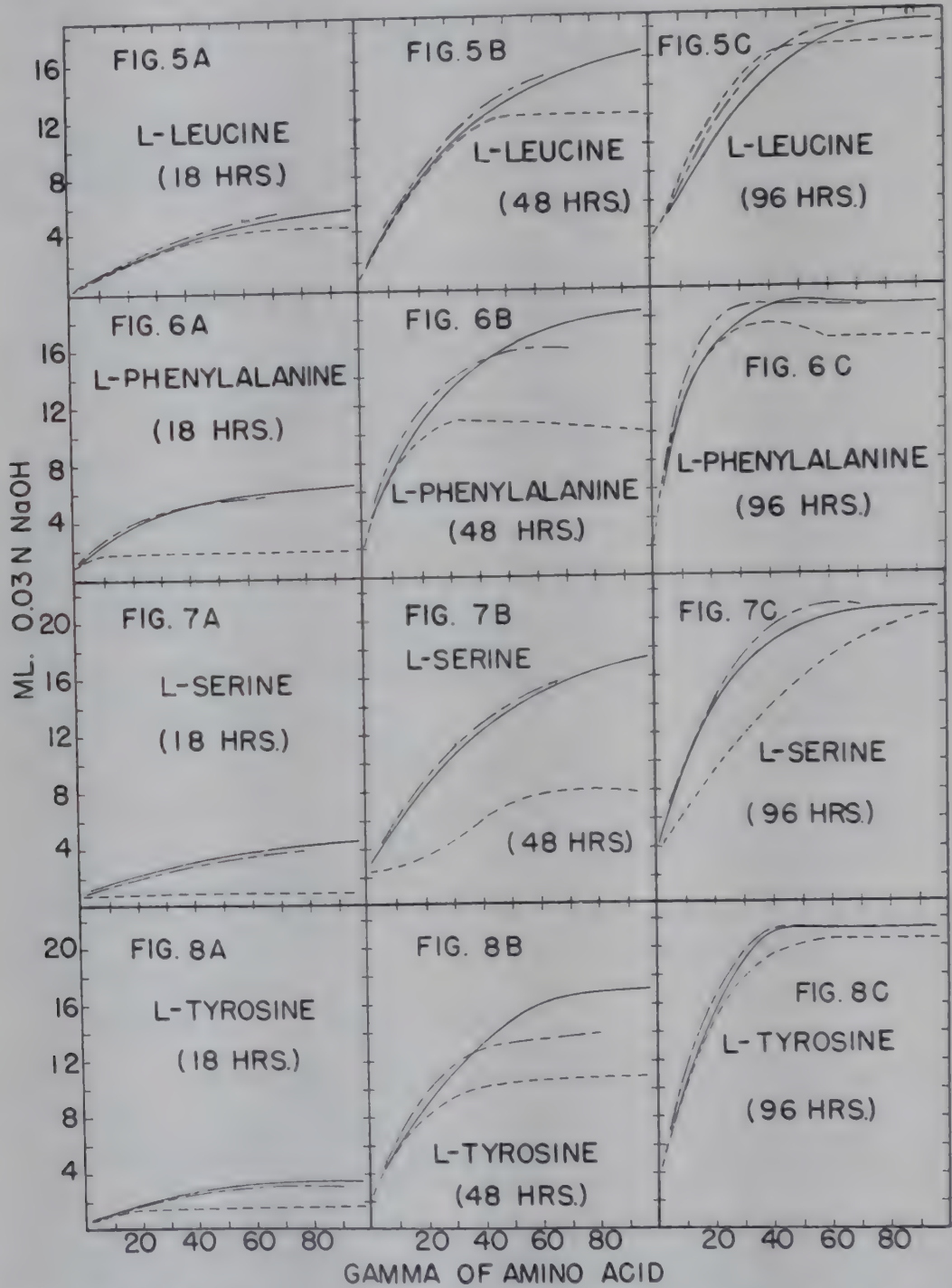
³ The activity of free and combined valine was not tested, owing to the lack of sufficient partial hydrolysate.



FIGS. 1, A to 4, C. Response curves of *L. casei* to free amino acids, partial hydrolysates of casein, and partial hydrolysates of bovine plasma albumin in a basal medium lacking a specified amino acid. The notations are as follows: short dash, free amino acid; long and short dash, partial hydrolysate of casein; solid curve, partial hydrolysate of bovine plasma albumin.

was calculated from its percentage in the completely hydrolyzed protein determined by microbiological assay (data given in Table I). The activ-

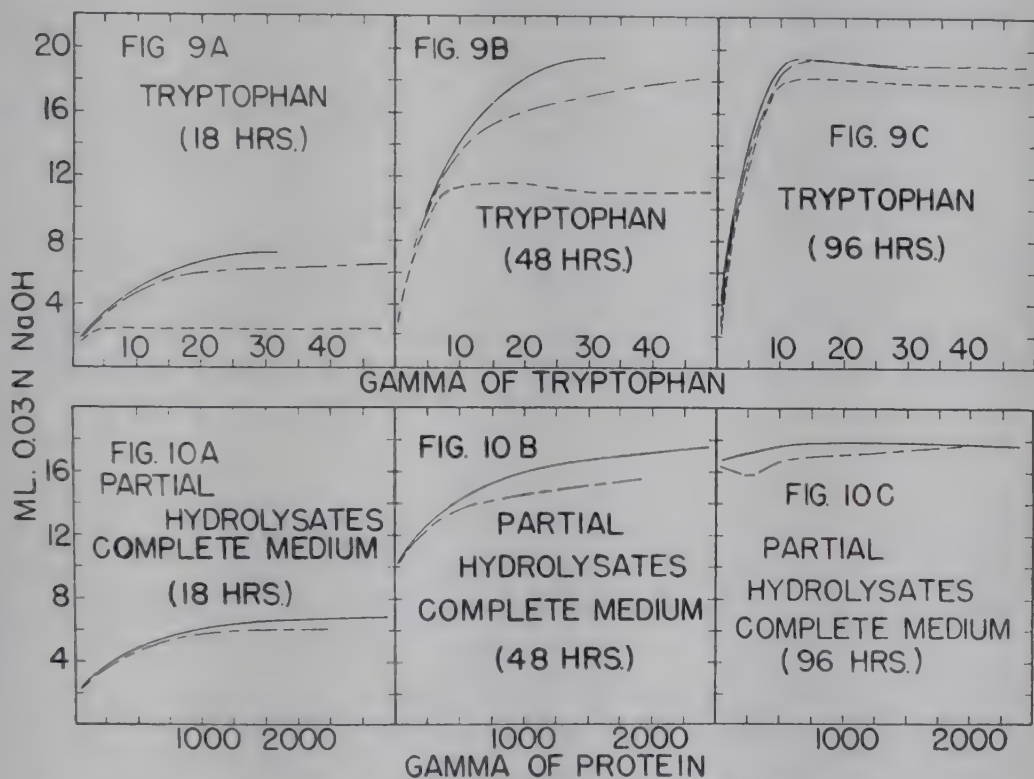
ity of the partial hydrolysates for *L. casei* grown on the complete basal medium was tested similarly in order to determine the over-all response



FIGS. 5, A to 8, C. The description is the same as that given for Figs. 1, A to 4, C.

of the organism. The results of these experiments are shown in Figs. 1, A to 10, C.

Chromatographic Experiments—Partial hydrolysates of bovine plasma albumin were separated into fractions by paper partition chromatography with the apparatus and techniques of Consden *et al.* (10). A 0.02 ml. volume of the partial hydrolysate approximately equivalent to 500 γ of the protein was placed on a sheet (18 $\times$ 22 inches) of Whatman No. 1 filter paper with the aid of a Kimble 0.2 ml. serological pipette. The solution was introduced in two equal aliquots in order to confine the spot to an area of about 1.5 cm. in diameter. The first aliquot was allowed



FIGS. 9, A to 10, C. The description is the same as that given for Figs. 1, A to 4, C, except that in Figs. 10, A to 10, C the complete basal medium was employed.

to dry before applying the second aliquot. The treated paper was equilibrated for 1 hour with water vapor saturated with phenol contained in a tight cabinet. Phenol saturated with water was placed in the tray and, when the solvent front reached the lower edge of the paper, the chromatogram was dried and developed by spraying the paper with a 0.1 per cent solution of ninhydrin in anhydrous *n*-butanol. There were five completely resolved spots, for which the R_f values were 0.16, 0.28, 0.43, 0.60, and 0.90. Six replicate chromatograms were prepared simultaneously on the same paper in order that one set might be developed for reference and the others retained undeveloped for subsequent use. Two-dimensional chromatograms were prepared similarly with the solvents, aqueous

phenol and *n*-butanol-benzyl alcohol or "collidine and aqueous phenol," but none of the spots moved detectably in the second dimension.

Determination of Distribution of Active Substances between Spots—The relative activity of each partial hydrolysate fraction was determined by using the basal medium lacking an essential amino acid. This procedure was repeated with the basal medium from which each other essential

TABLE II
R_F Values of Amino Acids

Amino acid*	Spot 5		Test mixture		Consden <i>et al.</i> (10)		Dent (12)
	Phenol	<i>n</i> -Butanol-benzyl alcohol	Phenol	<i>n</i> -Butanol-benzyl alcohol	Phenol	<i>n</i> -Butanol-benzyl alcohol	Phenol
?	0.04	0.00					
?	0.18	0.00	0.22	0.00			
Cystine†	0.33	0.22	0.31	0.25	0.13‡	0.00	
Serine	0.33	0.00	0.31	0.00	0.35	0.02	0.36
Glutamic acid	0.41	0.00	0.40	0.00	0.19	0.01	0.38–0.41
Threonine§	0.50	0.02			0.47	0.04	0.51
?	0.60	0.00	0.63	0.00			
Tyrosine	0.59	0.10	0.60	0.11	0.66	0.19	0.61
Valine	0.76	0.11	0.75	0.16	0.76	0.15	0.78
Isoleucine	0.80	0.15	0.81	0.22	0.87	0.27	0.83
Leucine	0.81	0.24	0.82	0.27¶	0.86	0.31	0.83
Phenylalanine	0.84**	0.25**	0.85**	0.31**	0.90	0.38	0.83**
Arginine	0.83	0.09	0.81	0.05	0.85	0.01	0.89

n-Butanol-benzyl alcohol is a water-saturated 1:1 mixture.

* Tryptophan did not appear in the chromatogram due probably to its low concentration and degree of destruction during hydrolysis.

† Develops bright yellow color, turning to pink on standing.

‡ Stated to be decomposition product.

§ Identified by test mixture containing threonine.

|| Characteristic dull gray-blue color with ninhydrin.

¶ Relatively high intensity compared to isoleucine.

** Characteristic greenish blue color with ninhydrin.

amino acid had been omitted in turn. An area corresponding to each of the five spots was cut out and placed in a 25 ml. glass-stoppered conical flask. 2 ml. of water and 5 ml. of the basal medium lacking an essential amino acid were added. The solution was autoclaved for 15 minutes at 15 pounds pressure and 0.05 ml. of a saline suspension of *L. casei* inoculum was added. The mixture was incubated for 18 hours at 35° and titrated with 0.016 N NaOH.

In the flasks containing Spots 1 to 4, acid production was approximately equal to the blank (about 4 ml. of 0.016 N NaOH), whereas in the flasks

containing Spot 5 it was significantly greater (1 to 6.3 ml. excess of 0.016 N NaOH) than the blank. It was concluded from this evidence that all of the active material in the partial hydrolysate was in Spot 5.

Determination of Amino Acids in Spot 5—Spot 5 of five paper strips was eluted essentially by the method of Dent (11), the combined eluates were evaporated to dryness, and the residue was dissolved in 0.5 ml. of 6 N HCl. The solution was heated in a sealed capillary tube for 14 hours at 15 pounds pressure and the cooled solution from the capillary tube was evaporated to dryness to remove excess HCl. The residue was dissolved in 0.1 ml. of water, and 0.02 ml. of the solution was placed on a sheet of Whatman No. 1 filter paper. The spot was neutralized with ammonia vapor and a chromatogram was obtained with water-saturated phenol as the solvent. The paper was dried in a current of air for 2 hours and at 130° for 20 minutes, and a chromatogram was obtained in the second dimension with a 1:1 water-saturated mixture of *n*-butanol and benzyl alcohol as the solvent. The R_F values of the chromatograms developed with ninhydrin, and those of the amino acids in a test mixture simulating the composition of the partial hydrolysate and treated similarly are shown in Table II.

DISCUSSION

The extreme fastidiousness of *L. casei* was first noted by Snell *et al.* (13), who employed acid-hydrolyzed casein and a fraction of liver or yeast as growth stimulants for this organism. Later investigators have postulated that the activity of the supplements might be due to glutamic acid (14, 15), glutamine (14, 16), asparagine (14, 17, 18), purines (19), pyrimidines (19), glutathione (20), oleic acid (21), and other substances (22–26).

Woolley and Sprince (27–34) have suggested that a peptide (strepogenin) or peptides in partial hydrolysates of proteins may serve as growth stimulants for *L. casei*. It was proposed that strepogenin contains glutamic acid and glycine, since the concentration of these amino acids increased proportionately in solutions of increasing strepogenin activity. DL-Seryl-glycyl-L-glutamic acid, the most active synthetic peptide tested, had the same activity per unit weight as a liver standard of a potency designated as 1. Woolley has pointed out, also, that strepogenin may be related to lycomarasmin, a substance which causes wilt in tomatoes and which was shown to contain asparagine, glycine, and α -hydroxypropionic acid. In other studies, Borsook *et al.* (35, 36) have isolated products from unhydrolyzed homogenates of liver and other organs, as well as from peptic digests of bovine serum albumin and other proteins, which were shown to be a single polypeptide or a mixture of polypeptides of similar composition, containing fifteen, and possibly, eighteen amino acids.

The fraction isolated chromatographically as Spot 5 from a partial hydrolysate of bovine plasma albumin in the present experiments appears to be homogeneous, since eluates, when rechromatographed, gave rise to only one spot with approximately the same R_F value as that found originally. It is entirely possible, however, that a mixture of peptides could respond in this manner. The partial hydrolysate has been subjected to larger scale fractionation on a column of cellulose fibers⁴ and a fraction corresponding to Spot 5, obtained on filter paper, has been isolated as a solid product. Further work on this product is in process. The amino acid composition of Spots 1 to 4, the response of other organisms to partial hydrolysates of proteins, and the influence of hydrolytic agents and conditions on the quantities of the fractions obtainable from different proteins are to be determined.

It is of particular interest that Woolley (32) has isolated peptides from pancreatic digests of dinitrophenylated insulin which, when hydrolyzed, yielded eight of the ten amino acids detected in the partial hydrolysates of bovine plasma albumin in the present experiments. Since a trypsinogen fraction was found to contain six of the same amino acids present in the insulin fraction, Woolley concluded that "a sizable piece of the two proteins is identical." Woolley (37), as well as earlier workers, has postulated that some proteins probably contain similar, if not identical, structural units.

The marked stimulatory effect of partial hydrolysates of casein and bovine plasma albumin on acid production by *L. casei* on a "complete" chemically defined medium is shown in Figs. 10, A to 10, C. That this over-all response of the organism may be the summation of a series of specific concurrent stimulations concerned with the utilization of the different residues of the essential amino acids bound in the peptide may be deduced from Figs. 1, A to 9, C and the data given in Table III. Acid production induced by the peptide varied markedly, depending upon the essential amino acid omitted from the basal medium. It was found, for example, that 40 γ of peptide-bound arginine in the partial hydrolysate of casein or bovine serum albumin exhibited activity towards *L. casei* equivalent to 100 γ of free arginine. Similarly, 1 γ of cystine in the casein hydrolysate was equivalent to 100 γ of free cystine. Furthermore, the stimulatory effects noted appear to be a function of the concentration of the bound amino acid in the partial hydrolysate rather than of the partial hydrolysate *per se*. For example, the ratio of the quantity of casein (1140 γ) supplying 40 γ of bound arginine equivalent in activity

⁴ Solka Floc BW-40, kindly supplied by the Brown Company, Berlin, New Hampshire, through the courtesy of D. H. McMurtrie. The authors are indebted to Dr. Harry F. Lewis, Paper Institute, Appleton, Wisconsin, for his assistance.

to 100 γ of free arginine to the quantity of casein (320 γ) supplying 1 γ of bound cystine equivalent in activity to 100 γ of free cystine was about 4:1.

These results oppose the hypothesis that the observed stimulatory effects may be due to some unknown factor present in the partial hydrolysate of the highly purified, crystalline sample of bovine serum albumin. It would appear more reasonable to assume that an increased rate of acid production resulted because *L. casei* utilized an essential amino acid more readily in the peptide-bound than in the free form. The higher (13 to

TABLE III

*Relative Activity towards L. casei of Free and Combined Amino Acids**

Amino acid	Amino acid in partial hydrolysate equivalent to 100 γ free amino acid		Relative activity of amino acid in partial hydrolysate		Protein containing micrograms of amino acid shown in Column A or B	
	Casein (A)	Bovine plasma albumin (B)	Casein†	Bovine plasma albumin‡	Casein	Bovine plasma albumin
	γ	γ			γ	γ
Arginine.....	40	40	2.5	2.5	1140	708
Cystine.....	1	2	100	50	320	30
Glutamic acid.....	100	150	1.0	0.66	480	670
Isoleucine.....	45	55	2.2	1.8	840	1910
Leucine.....	50	59	2.0	1.7	550	517
Phenylalanine.....	5	5	20	20	105	79
Serine.....	4	3	25	33	78	67
Tryptophan.....	2	2	25	25	155	328
Tyrosine.....	20	17	5	5.9	380	450

* Incubation time, 18 hours.

† 100/(per microgram of amino acid given in Column A).

‡ 100/(per microgram of amino acid given in Column B).

20 times) activity of glutathione than of cystine or cysteine observed by Riesen *et al.* (20) for *L. casei* has been interpreted in this manner. Tests (data not shown) made in the present experiments indicated that glutathione and cysteine had about the same activity towards *L. casei* calculated on an equivalent sulfur basis. It should be noted, however, that an oxidized peptone medium and 72 hours incubation were employed by Riesen *et al.* and an amino acid medium and 18 hours incubation by the present authors.

No information has been obtained in the present studies on the nature of the stimulatory peptide. Simmonds *et al.* (38), Krehl and Fruton (39), and Ågren (40, 41) have tested the streptogenin activity of a series of synthetic peptides containing from two to five of the amino acids ala-

nine, glutamic acid, glycine, leucine, serine, tyrosine, and valine. *L. casei* was stimulated slightly by L-serylglycyl-L-glutamic acid, but the other peptides were inactive or less active than free leucine or valine.

The three electrodialysis fractions of enzymatic hydrolysates of casein were shown by de Verdier and Ågren (42) and Ågren (43) to have streptogenin activity towards *L. casei*. The (anode) fraction having the highest activity was resolved with the aid of paper chromatography into two spots, of which only one contained growth-stimulating substances. The latter included free aspartic acid and a growth-stimulating seryl-glutamic acid peptide. The inactive spot contained a mixture of free glutamic acid and an asparagyl peptide probably containing serine, alanine, and valine.

SUMMARY

The response of *Lactobacillus casei* to partial hydrolysates of casein and bovine plasma albumin has been determined in terms of acid produced in a basal medium complete except for the omission in turn of each of the ten essential amino acids. In all cases but one, acid production was higher than that due to the parent amino acid tested similarly.

The partial hydrolysate of bovine plasma albumin was resolved into five spots with paper partition chromatography. The acid hydrolysate of the fastest running spot ($R_F = 0.90$) was shown by microbiological assay and paper chromatography to contain the ten amino acids essential for *L. casei*. Studies of Spot 5 are in process, although it has not been determined whether or not more than one peptide is present in this active fraction.

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ORTHOPHOSPHATE UPTAKE DURING THE OXIDATION OF FATTY ACIDS*

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It was demonstrated several years ago that the oxidation by tissue extracts of such metabolites as glucose, pyruvate, lactate, succinate, malate, and glutamate is accompanied by a net uptake of inorganic phosphorus (1-7). Various factors influencing the efficiency of these oxidative phosphorylations have been studied (1-12), and refinements in technique, permitting a direct estimate, have led to the demonstration that 2 to 4 moles of phosphate are fixed for each atom of oxygen consumed (9, 12).

A recent review (13) has called attention to the fact that "it has not been possible to demonstrate any net incorporation of inorganic phosphate into organic form during fatty acid oxidation." Lehninger and Kennedy (14) were unable to "demonstrate a *net* synthesis of newly esterified phosphate" with washed particulate matter of rat liver homogenate oxidizing octanoate. However, they did demonstrate, with the aid of radioactive phosphorus, that the oxidation of octanoate resulted in a greater fixation of P^{32} into the acid-soluble esters than was found to occur in control systems containing all additions except octanoate. Some years ago it was shown in this laboratory that a net uptake of orthophosphate occurs during the oxidation of endogenous phospholipid stores in epididymal spermatozoa (15). The uptake of inorganic phosphate was paralleled by an increase in the labile phosphate of the adenosine triphosphate (ATP) fraction.

In investigation of the mode of action of certain naturally occurring metabolic regulators, it was found that a net uptake of orthophosphate did occur during the oxidation of fatty acids by washed residue of rabbit kidney homogenate. This paper reports a study of the phosphate uptake associated with the oxidation of a variety of fatty acid substrates and the relationship of fluoride to reactions associated with the oxidation of "fat" and "carbohydrate" substrates.

EXPERIMENTAL

Respiration and phosphate uptake of washed particles from rabbit kidney were studied by techniques similar to those employed by Cross,

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Taggart, Covo, and Green (12). The enzyme preparation used corresponds to the "cyclophorase" R_3K preparation of Green *et al.* (16). Cytochrome *c* was found to increase the rate of respiration when relatively small quantities of the enzyme preparation were used as in the present work. It was therefore added routinely to all flasks.

Rabbit kidney cortex was homogenized in the Potter-Elvehjem apparatus in a 7:1 mixture of 0.15 M KCl-0.1 M NaHCO_3 and centrifuged at $1800 \times g$ (5°) for 5 minutes. The supernatant liquid phase was discarded. The residue was twice resuspended in 0.154 M KCl, centrifuged as above, and the supernatant liquids discarded. The final enzyme suspension was made with a volume of 0.154 M KCl equal to that of the sedimented residue.

The Warburg flask contents were as follows: main compartment 0.4 ml. of kidney particle suspension; 1.33×10^{-5} M cytochrome *c*; 6×10^{-5} M MgCl_2 ; 2×10^{-5} M ATP; 1.0×10^{-2} M KF; 7.34×10^{-5} M potassium phosphate buffer at pH 7.3; 2.9×10^{-4} M potassium fumarate. The side arms contained 0.05 ml. of a yeast hexokinase preparation and 0.1 ml. of 0.15 M glucose. Sodium pyruvate was 1.67×10^{-2} M in the "carbohydrate" experiments and the amounts of the fat substrates are indicated in Table I. The final volume was always made up to 3.0 ml. with 0.154 M KCl. KOH and a filter paper leaf were used in the center wells to absorb CO_2 .

The hexokinase-glucose system as used by Cross *et al.* (12) was found to be very effective for trapping high energy phosphate by converting it to hexose phosphate and was used in all these experiments. The yeast hexokinase preparation was an active suspension obtained at Step 3a according to the procedure of Berger, Slein, Colowick, and Cori (17). Maximum PO_4 transfer was obtained when 0.02 to 0.05 ml. of this preparation was used. The flasks were chilled and kept in an ice bath until the enzyme preparations had been added, then attached to the manometers and equilibrated for 5 minutes in the water bath (30°) before the taps were closed. After initial readings had been taken, the side arm contents of all the flasks were tipped in. A zero time flask was removed to the ice bath immediately and deproteinized with 2 ml. of cold 17.5 per cent trichloroacetic acid. Enzymatic oxidation in the presence of hexokinase was allowed to proceed for 10 minutes in the remaining flasks. After final readings had been taken, the flasks were quickly removed to the ice bath and enzymatic action stopped with trichloroacetic acid as above. The deproteinized flask contents were analyzed for orthophosphate by the procedure of Lowry and Lopez (18). The difference in inorganic orthophosphate between zero time flasks and those incubated 10 minutes longer is the net uptake of phosphate which occurs concomitantly with the measured respiration.

RESULTS AND DISCUSSION

Studies of phosphorylations associated with oxidation of fatty acids are complicated by two major difficulties. First, it is necessary to carry out fatty acid oxidation concomitantly with the oxidation of a catalytic quantity of some dicarboxylic acid (19) to allow the Krebs cycle to function as a channel for the complete oxidation of the fatty acid. In the present work, each experiment included control flasks containing all co-factor additions and a catalytic quantity of fumarate but no other oxidizable substrate. Secondly, fluoride may not be used to inhibit phosphatase activity since it strongly inhibits fatty acid oxidation (19, 20). While fluoride depresses "carbohydrate" oxidation considerably, it permits retention of esterified phosphate (1, 6, 9, 10) and is required to demonstrate maximum P:O ratios (9, 12).

It was realized during the progress of these experiments that the use of a 10 minute interval as being representative of continuous enzymatic processes may lead to fallacious conclusions. Longer experiments were run to determine whether the reactions under study were merely short term processes or whether they could be maintained for longer periods. The data shown in Fig. 1 are typical of experiments in which oxidation was allowed to proceed for 70 minutes. Phosphorylation was not studied, although other experiments indicate that phosphate uptake continues as long as the hexokinase is active and there is sufficient glucose and inorganic orthophosphate. The linearity of the respiration curves justified taking the oxygen uptake and phosphorylation of the first 10 minutes as representative of the functional activity of the system.

Under the conditions of our experiments, fluoride (0.01 M) completely abolished the respiration increment which ordinarily occurs when fatty acids are added to the otherwise complete enzyme system. In agreement with the results of many previous workers, fluoride depressed the respiration with pyruvate and β -hydroxybutyrate as the substrates. These results are emphasized here since they bear on the phosphorylation results which follow.

After our preliminary observations a detailed study was made of the rates of respiration and phosphate uptake in the presence and absence of fluoride. The results of typical experiments are shown in Table I and a summary of all experiments is presented in Table II. In addition to the control flasks containing only a trace of fumarate, additional control flasks containing the complete system for pyruvate oxidation were included in each experiment.

It is clearly demonstrated that the oxidation of each of the fatty acids (acetate, butyrate, crotonate, caprylate) is accompanied by a *net* phosphate uptake far greater than that which occurs with the fumarate alone.

In the presence of fluoride, which abolishes the oxidation of these substrates, the phosphate uptake in the presence of the fatty acids is **not** significantly different from that occurring in the control vessels without fatty acids.

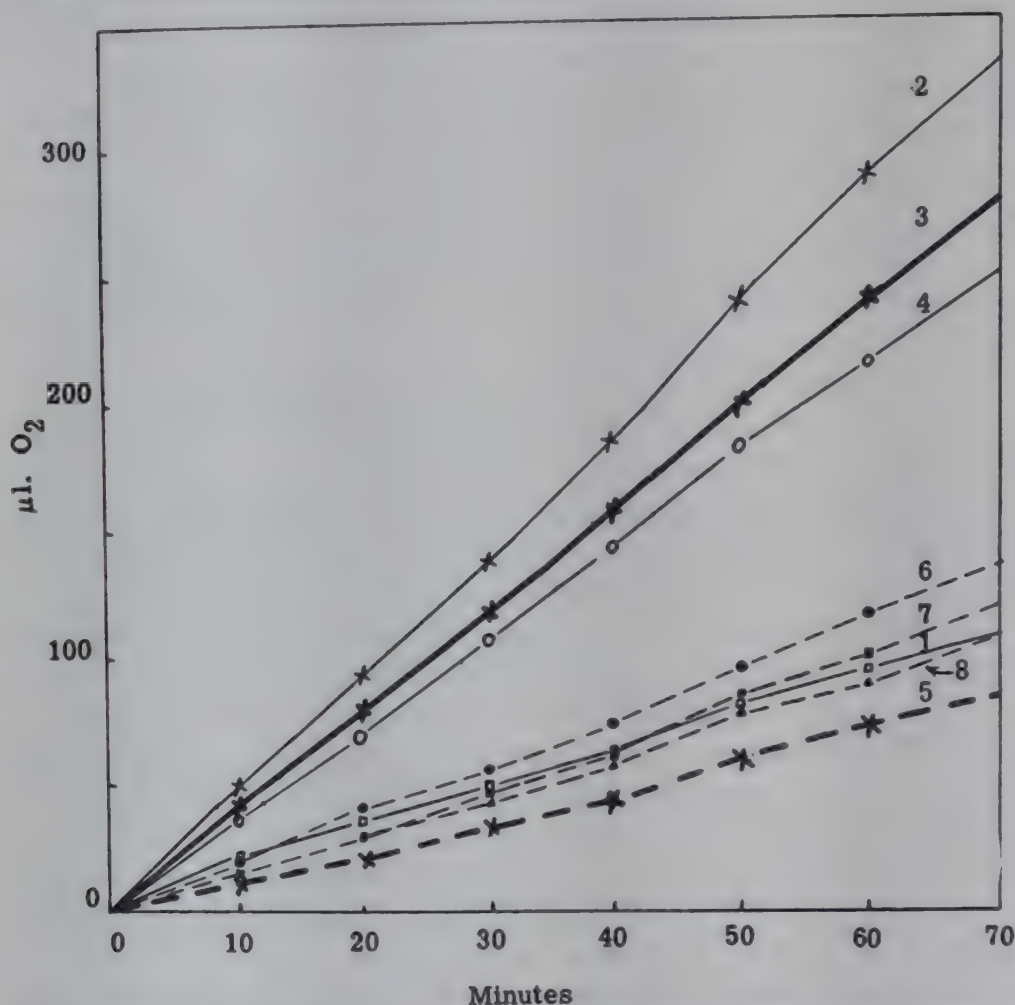


FIG. 1. Rates of oxygen consumption by washed residue of rabbit kidney homogenate. Curves 1 to 4, no fluoride added; Curves 5 to 8, 0.01 M fluoride present. Curves 3 and 5 represent the results obtained with several individual substrates which are oxidized at approximately the same rate. Curve 1, 2.9×10^{-4} M fumarate; Curve 2, acetate; Curve 3, pyruvate, butyrate, β -hydroxybutyrate, or crotonate; Curve 4, acetoacetate; Curve 5, fumarate alone, acetate, butyrate, or crotonate; Curve 6, pyruvate; Curve 7, β -hydroxybutyrate; Curve 8, acetoacetate. The substrate concentration is recorded in Table I.

The metabolism of acetoacetate and β -hydroxybutyrate resembles that of pyruvate. The oxidation of these substrates is accompanied by phosphate uptake both in the presence and absence of fluoride. It is possible that the phosphate uptake accompanying the oxidation of β -hydroxybutyrate is coupled with the electron transport resulting from its one-step

oxidation to acetoacetate (21) and not with the complete combustion of the carbon chain. This one-step oxidation was reported in 1945 (22) to furnish energy for the motility of malonate-poisoned spermatozoa which cannot oxidize acetoacetate and recently was shown by Lehninger (21) to increase the fixation of P^{32} from orthophosphate by rat liver preparations which likewise do not oxidize acetoacetate. However, acetoacetate is oxidized by the rabbit kidney preparation (Tables I and II) with a net uptake of phosphate both in the presence and absence of fluoride. When fluoride is present to inhibit phosphatase activity, each of these substrates

TABLE I

Uptake of Orthophosphate Accompanying Oxidation of Various Fatty Acids and of Pyruvate

Experiment	Fluoride present	Control, 0.87 μ M fumarate			Fatty acid				"Carbohydrate" control, 50 μ M pyruvate		
		μ M O ₂ consumed	μ M P uptake	P:O ratio		μ M O ₂ consumed	μ M P uptake	P:O ratio	μ M O ₂ consumed	μ M P uptake	P:O ratio
A	0.01 M	1.0	3.1	1.6	30 μ M acetate	0.9	3.3	1.8	1.8	9.8	2.7
	None	2.3	2.7	0.6		2.5	6.7	1.6	3.3	9.3	1.4
B	0.01 M	0.9	4.0	2.4	30 " butyrate	1.5	6.2	2.1	2.6	13.6	2.6
	None	1.9	1.2	0.3		3.6	9.1	1.3	4.4	12.4	1.4
C	0.01 M	1.1	3.6	1.6	2.5 " caprylate	0.7	2.9	2.1	1.6	11.8	3.7
	None	1.5	1.3	0.4		4.0	10.6	1.3	4.6	13.5	1.5
D	0.01 M	0.7	2.9	2.2	15 " crotonate	1.0	3.5	1.7	2.5	13.9	2.8
	None	0	0	0		2.7	8.2	1.5	4.7	14.2	1.5
E	0.01 M	0.7	3.3	2.5	15 " β -hydroxybutyrate	1.8	11.3	3.2	2.3	10.2	2.2
	None	2.0	0	0		3.8	10.8	1.4	4.8	14.8	1.5
F	0.01 M	0.7	7.4	5.3*	30 " acetoacetate	1.7	11.4	3.4	2.1	13.0	3.1
	None	1.6	5.3	1.7		3.4	9.9	1.5	4.7	13.0	1.4

* The low respiratory rate does not permit an accurate estimate of the P:O ratio.

is oxidized with relatively high P:O ratios. In view of the relatively small number of experiments with β -hydroxybutyrate, the P:O ratio of 2.3 is not significantly lower than the value of 3 obtained with pyruvate and acetoacetate.

With pyruvate as the substrate, approximately equal amounts of phosphate were fixed in the presence and absence of fluoride. With both acetoacetate and β -hydroxybutyrate the highest phosphate uptake consistently occurred in the presence of fluoride (Table II). As pointed out previously, the fatty acids were oxidized only in the absence of fluoride and only under this condition was phosphate uptake greater than in the

control.¹ It should be pointed out that, in the absence of fluoride, the P:O ratios for all substrates were approximately the same (1.0 to 1.7). It seems, therefore, that, if an agent could be obtained which possessed the phosphatase-inhibiting properties of fluoride and yet did not inhibit fatty acid oxidation, it might be possible to demonstrate P:O ratios for fat oxidation as high as those accompanying pyruvate oxidation.

Thus far, all attempts to demonstrate a net uptake of orthophosphate during the oxidation of fatty acids by normal rat liver and kidney prep-

TABLE II

Effect of Fluoride on Phosphorylations Associated with Oxidation of Various Substrates

Substrate*	F concentration	No. of experiments	Micromoles P uptake Microatoms O ₂ consumed		P uptake with F P uptake without F	
			Range	Average $\pm$ s.d.	Range	Average $\pm$ s.d.
	<i>M</i>					
Pyruvate	0.01	23	1.4-4.9	2.9 $\pm$ 0.62	0.69-2.29	1.13 $\pm$ 0.35
	0.0	23	0.6-1.9	1.3 $\pm$ 0.35		
Acetoacetate	0.01	5	2.5-4.0	3.1 $\pm$ 0.56	1.05-2.07	1.36 $\pm$ 0.32
	0.0	6	0.8-2.1	1.5 $\pm$ 0.74		
β -Hydroxybutyrate	0.01	4	1.7-3.2	2.3 $\pm$ 0.53	1.05-3.46	1.71 $\pm$ 0.75
	0.0	4	0.3-1.4	1.0 $\pm$ 0.26		
Acetate	0.01	4	1.2-2.1	1.7 $\pm$ 0.32	0.44-0.76	0.56 $\pm$ 0.11
	0.0	5	0.9-1.7	1.3 $\pm$ 0.35		
Butyrate	0.01	2	1.2-2.1	1.6	0.54-0.68	0.61
	0.0	2	0.8-1.3	1.0		
Caprylate	0.01	2	1.2-2.1	1.6	0.27-0.55	0.41
	0.0	2	0.8-1.3	1.0		
Crotonate	0.01	3	1.7-4.0	2.8 $\pm$ 0.95	0.43-0.74	0.61 $\pm$ 0.13
	0.0	4	1.5-2.4	1.7 $\pm$ 0.37		
Fumarate control	0.01	14	1.3-5.3	2.8 $\pm$ 1.16	0.86-3.32	1.07 $\pm$ 0.91
	0.0	14	0.3-2.1	1.1 $\pm$ 0.56		

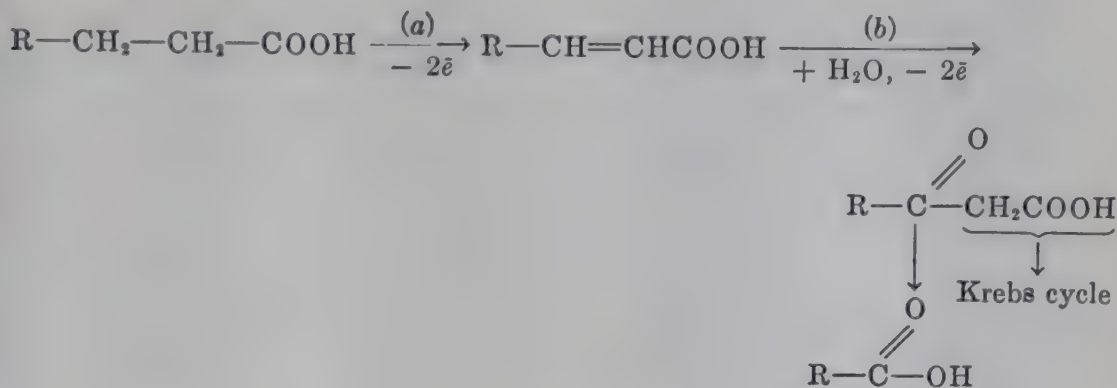
* Substrate concentrations as in Table I.

arations have been as unsuccessful in our hands as have the results reported by others. The washed preparation of rabbit kidney appears to retain much less phosphatase activity than do rat tissue preparations.

Site of Fluoride Inhibition of Fatty Acid Oxidation—The data obtained provide some evidence that fluoride inhibits fatty acid oxidation at some step prior to the formation of β -keto acids and subsequent to the oxida-

¹ Higher phosphate uptake in the absence of fluoride during fatty acid oxidation than during the oxidation of fumarate alone was substantiated by statistical analysis in which a *T* value of 4.38 was obtained.

tion of the unsaturated fatty acid. If one assumes the oxidation to proceed as follows:



fluoride probably exerts its inhibition on the reactions represented by (b), since the oxidation of crotonate but not of acetoacetate is inhibited by fluoride. The fatty acid dehydrogenase of Lang (23) is not inhibited by fluoride.

Lipmann and Perlmann (24) have suggested that the oxidation of the unsaturated acids may be accompanied by the simultaneous addition of a substituent such as phosphate. In the hope of throwing further light on the mechanism of reaction (b) and on the site of fluoride inhibition, studies were made with the phosphoric ester of DL- β -hydroxybutyrate as the substrate. This ester was synthesized by Dr. Mann (25). In the presence of fluoride the synthetic ester was not oxidized. In the absence of fluoride, its oxidation and the accompanying phosphate uptake were about the same as when β -hydroxybutyrate was the substrate. It is possible that it was oxidized only after having been hydrolyzed to free β -hydroxybutyric acid. These results do not exclude the possibility of phosphorylation taking place at some other point in the molecule and certainly do not render less tenable the original postulate of Lipmann and Perlmann (24).

SUMMARY

1. The oxidation of acetate, butyrate, caprylate, and crotonate by washed residue of rabbit kidney homogenate in the absence of fluoride is accompanied by a *net* uptake of orthophosphate. Under the conditions described P:O ratios of 1.0 to 1.7 were obtained.

2. The oxidation of pyruvate, acetoacetate, and β -hydroxybutyrate in the same system led to P:O ratios of about 3 in the presence of fluoride and of 1.0 to 1.5 in the absence of fluoride.

3. Evidence concerning the site of fluoride inhibition of fatty acid oxidation is presented and discussed.

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SYSTEMS FOR THE SEPARATION OF PHOSPHORIC ESTERS BY SOLVENT DISTRIBUTION*

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The isolation and purification of phosphoric esters are usually accomplished by the fractional precipitation of their metallic salts. This method has been extremely useful and has led to the isolation of a large number of biologically important phosphorus compounds. However, some types of compounds are merely separated by this procedure into groups from which the individual members cannot be readily isolated.

The application of solvent extraction, particularly of the Craig counter-current distribution type (1), appeared to be a desirable approach to this problem. However, the failure of most of the phosphoric esters of biological importance to be distributed to any appreciable extent between an aqueous phase and an organic solvent seemed to preclude the application of this principle, unless a way could be found to increase the solubility of these compounds in the organic solvent phase. A similar difficulty had been experienced in the attempt to apply solvent distribution to the separation and purification of the streptomycins, basic compounds with a large carbohydrate moiety which rendered them insoluble in non-polar liquids. This obstacle was overcome by Plaut and McCormack (2) who successfully separated and determined streptomycin types with a solvent pair which contained amyl alcohol and stearic acid in one phase and aqueous buffer in the other.

By analogy it seemed possible to increase the solubility of phosphoric esters (acidic substances) in organic solvents by the inclusion of a long chain fatty amine in the system.

Methods

1 per cent aqueous sodium chloride solution and organic solvent were mutually saturated. 5 gm. of a long chain fatty amine were then dissolved with heating in 100 ml. of the organic solvent layer. The aqueous and organic solvent phases were mixed and acid was added until the

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desired pH was obtained.¹ The composition of the various systems is shown in Table I.

When the distribution coefficient was determined by the direct method, the phosphoric ester was dissolved in the aqueous phase and distributed against an equal volume of the solvent phase. The compound tested was determined in the aqueous phase before and after partition. The amount of the material in the solvent was ascertained by difference.

Craig counter-current distributions were carried out in the usual manner (1) with 8 ml. of each phase per tube. After the distribution was

TABLE I
Composition of Solvent Systems

	System 1	System 2	System 5	System 6	System 24	System 24a
	pH 7.5	pH 7.5	pH 7.4	pH 7.05	pH 7.1	pH 7.2
Solvent phase	<i>n</i> -Amyl alcohol	<i>n</i> -Amyl alcohol	<i>n</i> -Butanol	<i>n</i> -Hexanol (Eastman practical)	<i>n</i> -Octanol (Eastman practical 90%+)	<i>n</i> -Octanol (Eastman primary)
Aqueous phase	Armeen 18D 0.5 gm. citric acid per 100 ml.*	Armeen 16D 0.5 gm. citric acid per 100 ml.*	Armeen 16D 0.5 ml. HCl per 100 ml.†	Armeen 18D 0.5 ml. HCl per 100 ml.†	Armeen 18D 0.5 ml. HCl per 100 ml.†	Armeen 18D 1.2 ml. HCl per 100 ml.†

The different samples of Armeen required varying amounts of acid for pH adjustment.

* $C_6H_8O_7 \cdot H_2O$.

† Concentrated HCl.

finished, the tubes were completely emptied into test-tubes by means of a siphon-vacuum arrangement. The compound was completely displaced into the aqueous phase by the addition of 0.4 ml. of concentrated ammonium hydroxide to each tube and vigorous agitation for 30 minutes in a mechanical shaker.

¹ The solutions of Armeen 18D in hexanol and octanol tend to become cloudy if allowed to stand several hours; however, if the alcohol is previously saturated with the aqueous phase and if the aqueous phase is then added to the organic solvent phase immediately upon cooling, the total system (*i.e.*, both phases in mutual contact) is stabilized. For work with labile compounds at low temperatures, it is necessary to use shorter chain amines (C_3 to C_{11}).

Total phosphorus was determined by the method of King (3), inorganic orthophosphate according to Fiske and Subbarow (4), and easily hydrolyzable phosphorus by hydrolysis for 10 minutes at 100° in 1 N hydrochloric acid prior to phosphorus assay. Pentose was assayed by the procedure of Meijbaum (5); however, it was found necessary to prolong the heating time from 20 to 30 minutes to obtain maximum color development.

Materials²

Glucose-6-phosphate (6), sorbose-1-phosphate and sorbose-6-phosphate (7), propanediol phosphate (8), and L- α -glycerophosphate (9) were synthesized by the methods referred to. Adenosine triphosphate (ATP) (93 to 98 per cent pure) was isolated from rabbit muscle (10). Fructose-6-phosphate was prepared from hexose diphosphate (11), adenosine diphosphate (ADP) (90 to 96 per cent pure) was prepared from ATP by treatment with yeast hexokinase and glucose and isolated as described by LePage (10), adenosine-5-phosphate (muscle adenylic acid) was prepared by alkaline hydrolysis of ATP (12), and glucose-1-phosphate (98 per cent pure) was prepared according to McCready and Hassid (13). Diphosphopyridine nucleotide (70 per cent) was isolated from yeast according to LePage (14). Triphosphopyridine nucleotide (67 per cent pure) (15) was obtained from Dr. G. Mueller. The 3-phosphoglyceric acid was prepared according to Neuberg and Lustig (16). Yeast adenylic acid (3-phosphate), guanylic acid, cytidylic acid, β -glycerophosphate (Malinckrodt), and hexose diphosphate were obtained from commercial sources.

Hexadecylamine (Armeen 16D) and octadecylamine (Armeen 18D) were gifts of Armour and Company, Chicago, Illinois.

RESULTS AND DISCUSSION

The partition coefficients of several phosphorus compounds were determined in a number of different solvent pairs. The values of the coefficients are reported to the nearest significant figure, and, in those cases in which variable results were obtained, the range of values is shown. The data in Table II indicate that certain of these substances can be extracted and separated successfully by these systems.

The distribution coefficients which were obtained for adenosine-5-phosphate, ADP, and ATP in System 24, for example, are sufficiently different to permit a successful separation of these compounds. This seems to be the first procedure which has promise for the practical separation of ATP and ADP.

² We wish to thank Dr. K. M. Mann who synthesized the sorbose esters and Mr. George Lampson who prepared the propanediol phosphate used in this work.

The separation of triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN) was accomplished by Warburg *et al.* (17) and, somewhat more readily, by the recent procedure of LePage and Mueller (15) in which these nucleotides are separated by chromatography on charcoal. Hogeboom and Barry (18) purified DPN by counter-current distribution in a system containing phenol; however, TPN was not examined. Since DPN is not extracted in System 24a, while TPN has a

TABLE II
*Distribution Coefficients (K) of Phosphoric Esters**

Substance	System 1	System 2	System 5	System 6	System 24	System 24a
Pyrophosphate	>40	42	2.0	5.4	>45	
Orthophosphate	0.3	0.6	0.1	0.3	1.5	
ATP	11	13.5	1.4	15	43.2	
ADP	7		0.3	5.1	7.8	
5-Adenylic acid	0.4		0.2	0.5	1.6-2.2	2.7
3-Adenylic "	0.8		0.3	0.6	0.6	1.5
Cytidylic "	2.7		0.7	0.7		2.5
Guanylic "	0.4		0.2	0.4		4.8
Glucose-1-phosphate	0.1	0.1	0.0	0.0	0.0	0.1
Glucose-6-phosphate	0.1		0.09	0.4		0.8
Sorbose-1-phosphate	0.04		0.5	0.1		0.8
Sorbose-6-phosphate	0.9		0.1	1.8		2.4
Fructose-6-phosphate	0.2		0.03	0.15		0.7
Hexose diphosphate	1-2.8		1.0	0.7	3.3	
Phosphoglycerate	1.3		0.4	0.3	0.9	
β -Glycerophosphate	1.1		0.2	0.9		1.9
L- α -Glycerophosphate	1.6		0.2	1.5		2.7
Serine phosphate	0.6					
DPN	0.0		0.1	0.0		0.0
TPN	0.2		0.2	0.2		0.6
Cocarcboxylase	0.7					
Propane diolphosphate						0.3

* K = ratio of concentration in solvent layer per concentration in aqueous layer.

distribution coefficient of 0.6, the separation of these substances can be easily achieved by the use of this solvent pair.

Phosphoglyceric acid and hexose diphosphate are another pair of phosphoric esters which are difficult to separate by presently available procedures. The partition coefficients of these substances in System 24 are sufficiently different to make possible their separation if an adequate number of plates is used.

Striking differences in distribution coefficients were observed in the case of some of the hexose monophosphates. For example, glucose-6-

phosphate and fructose-6-phosphate have partition coefficients 8 times larger than that of glucose-1-phosphate, and that of sorbose-6-phosphate is even larger in System 24a.

The differences in distribution coefficients of yeast adenylic, muscle adenylic, cytidylic, and guanylic acids in Systems 1 and 24 may make possible the separation of these substances if a large number of plates is used.

The possibility of using solvent distribution for the separation of phosphoric esters is enhanced by the difference in selectivity which was observed between certain solvent pairs. For example, there is a 3-fold difference between the distribution coefficients of glucose-6-phosphate and fructose-6-phosphate in System 6, while they are practically the same in System 24a. Sorbose-6-phosphate had a coefficient 18 times higher

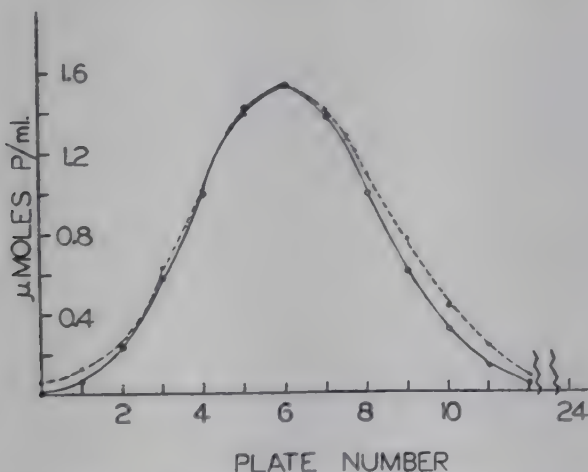


FIG. 1. Counter-current distribution of adenosine-3-phosphate in System 5. X, analytical values; O, theoretical curve for $K = 0.34$.

than that of sorbose-1-phosphate in System 6, while in System 5 that of sorbose-1-phosphate is 5 times larger than that of sorbose-6-phosphate. This makes it seem likely that in extending this principle other solvent systems may be found which are even more favorable for certain separations than those studied here.

When yeast adenylic acid was partitioned in System 5 by the counter-current distribution method of Craig (1), a curve was obtained which deviated only slightly from "normal." This small difference from the theoretical curve is probably due to impurities in the preparation. This is borne out by the finding that the adenine content of this sample of adenylic acid was about 5 per cent lower than its organic phosphate and pentose content (Fig. 1). This, together with the observation that the partition coefficient of ATP remained constant when tested over a range

of concentrations from 0.0012 M to 0.012 M in System 5, indicates that the distribution of phosphoric esters in these systems is not complicated by molecular association.

In agreement with earlier work on streptomycin (2) a sharp variation of the distribution coefficient with pH was found (Fig. 2). However, the coefficient remained constant in the buffered ranges (about pH 7.1 and 7.3 for System 24). Therefore, for the sake of best reproducibility, in our solvent pairs that pH was chosen at which the buffering capacity was maximum.

It is possible that the values for some of the partition coefficients reported here will have to be revised in the future, since it is already apparent from distribution studies now in progress that some of the phos-

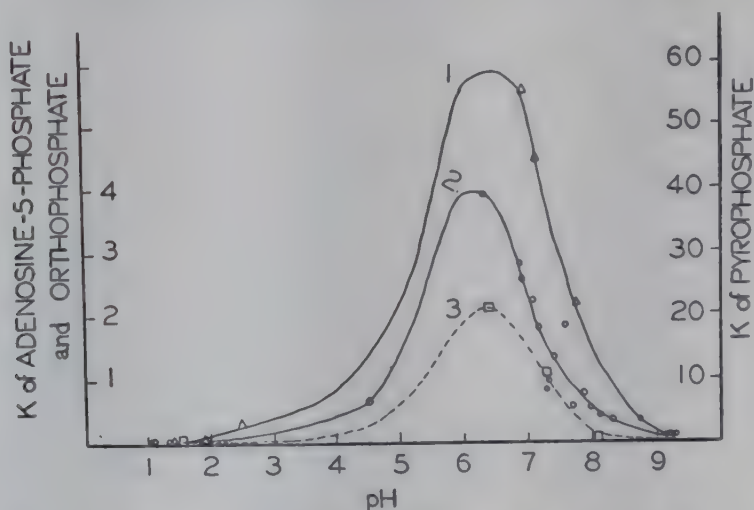


FIG. 2. Effect of pH on distribution coefficient for System 24. Curve 1, pyrophosphate; Curve 2, adenosine-5-phosphate; Curve 3, orthophosphate.

phoric esters isolated from biological materials are not as homogeneous as would appear from conventional testing methods.

SUMMARY

Biologically important phosphoric esters can be effectively distributed between an organic solvent and an aqueous phase when a long chain fatty amine is included in such a system. The fatty amines seem to increase the solubility of phosphoric esters in the less polar phase.

The distribution coefficients of a variety of phosphoric esters were studied in a number of such solvent pairs. The partition of many of these substances is sufficiently different to permit simple separations which were difficult to achieve with previous methods.

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EVIDENCE FOR A CALCIUM REQUIREMENT FOR *LACTOBACILLUS DELBRUECKII* 9595

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Earlier work (1) on the mineral requirements of a group of lactic acid bacteria showed that Ca^{++} was not essential for growth. *Lactobacillus delbrueckii* 9595 was not included in this group. Henderson and Snell (2) found that 2 per cent Salts C (3) supplied sufficient Mg^{++} , Mn^{++} , and Fe^{++} for good growth, even in the presence of 2 per cent sodium citrate buffer. However, no mention was made of serine assay conditions.

In the present investigation with *L. delbrueckii* and limiting serine conditions it was possible to demonstrate a citrate toxicity which was not removed completely by either Mg^{++} or Mn^{++} or a combination of these two cations. However, the addition of Ca^{++} had a pronounced effect. The data to be presented will show the nature of the effect of Ca^{++} on the growth of *L. delbrueckii* with different limiting amino acid conditions and also with different buffers.

EXPERIMENTAL

Materials—The salts employed in this study were all c.p. or of reagent grade. The cations Mg^{++} , Mn^{++} , and Ca^{++} were added as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

Methods—The experimental technique employed and the cultures of the microorganisms used were the same as those described previously (4). The media of Henderson and Snell (2) and Stokes, Gunness, Dwyer, and Caswell (5) or modifications of these two media were employed in the present investigation.

Results

Comparisons of the medium of Henderson and Snell (2) with the medium of Stokes *et al.* (5), both free of threonine, showed that the medium of Henderson and Snell exerted a marked depression on the growth response of *L. delbrueckii* to low levels of serine (0 to 100 γ of the DL form). Screening experiments on the components of the medium of Henderson and Snell pointed to sodium citrate as the constituent responsible for the growth depression.

Further investigations disclosed that neither Mg^{++} nor Mn^{++} in amounts

up to 10 mg. per 10 ml. tube overcame completely the toxicity exerted by the citrate buffer; however, growth was increased slightly as the amounts of Mg^{++} and Mn^{++} were increased. Increasing amounts of Salts C gave the same type of effect as was obtained with the addition of either Mg^{++} or Mn^{++} or a combination of these two cations. The slight increases in growth obtained with increasingly larger amounts of Mg^{++} and Mn^{++} suggested either a sparing action of these two cations on some active constituent or impurity in the medium, or the salts employed to supply the above two cations contained an active impurity.

Effect of Ca^{++} on Growth Responses with Citrate Buffer and Varying Amounts of Serine and Threonine—As indicated by Fig. 1, Ca^{++} has a pro-

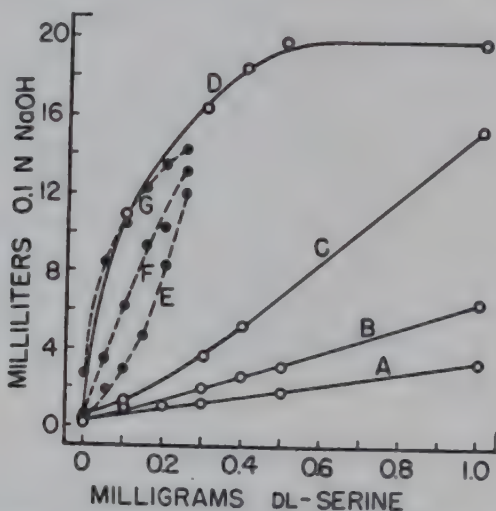


FIG. 1. Standard serine curves as influenced by added calcium. Curves A and E, no Ca^{++} added; Curves B and F, 0.2 mg. of Ca^{++} added; Curves C and G, 1.0 mg. of Ca^{++} added; Curve D, 10 mg. of Ca^{++} added; broken line curves, no threonine; solid line curves, 2 mg. of DL-threonine per 10 ml. tube. Basal medium, amino acids of Stokes *et al.* (5), and other constituents as in the medium of Henderson and Snell (2), except 2 γ instead of 10 γ of calcium pantothenate.

nounced effect on the growth response of *L. delbrueckii* to serine. Also to be noted from these data is the increased amount of Ca^{++} required in the presence of threonine. 10 mg. of Ca^{++} were required to give essentially the same growth response in the presence of threonine as was obtained with 1 mg. of Ca^{++} in the absence of threonine.

Further studies with levels of DL-serine up to 8 mg. per 10 ml. tube showed that both the stimulatory effect of added Ca^{++} and the inhibitory effect of threonine decreased as the serine level was increased. However, growth obtained in tubes containing Ca^{++} was always higher than in comparable tubes containing no Ca^{++} .

Effect of Ca^{++} with Limiting Amino Acid Conditions Other Than Serine—

With assay levels of arginine, leucine, and valine (Table I), the growth stimulation caused by Ca^{++} seems to be a function of the level of serine in the medium, especially with threonine present. With 2 mg. of DL-serine per tube (a level employed in many assay media) larger differences in growth between tubes with and without Ca^{++} were observed than in tubes containing 4 and 8 mg. of DL-serine. Thus from these data it seems that Ca^{++} is most closely associated with serine utilization.

The extent of growth also influences the amount of stimulation obtained from added Ca^{++} (Fig. 2). With 50 γ of DL-leucine (Curve G, Fig. 2), Ca^{++} caused only a slight stimulation at growths equivalent to approximately 4 ml. of 0.1 N NaOH. Leucine at this level was the primary limiting factor

TABLE I

Influence of Level of Serine on Growth Stimulation by Calcium with Different Limiting Amino Acids

Basal medium same as for Fig 1.

DL-Serine	DL-Threonine	L-Arginine monohydrochloride 50 γ		DL-Leucine 100 γ		DL-Valine 100 γ	
		Ca ⁺⁺ added					
		0 mg.	0.2 mg.	0 mg.	0.2 mg.	0 mg.	0.2 mg.
		Titration, ml. of 0.1 N NaOH					
mg.	mg.						
2	0	5.95	7.40	10.00	11.87	9.20	11.50
2	2	3.84	6.00	6.10	8.93	6.08	7.90
4	0	6.07	7.65	10.74	12.55	10.90	10.73
4	2	6.10	6.92	9.90	11.30	6.88	8.42
8	0	6.08	7.85	11.50	11.85	10.00	10.48
8	2	6.32	6.74	10.20	11.46	7.34	8.50

rather than the 2 mg. of serine present in the basal medium. Greater stimulation of growth by Ca^{++} as the leucine concentration was increased (Curves H, I, and J of Fig. 2) seems to be a result of a sparing action of Ca^{++} on the serine of the medium. All these data suggest that Ca^{++} is associated only with the serine utilization of *L. delbrueckii*.

Ca⁺⁺ Studies with Different Buffers—A comparison of acetate, succinate, and citrate as buffers (Table II) indicates that with citrate the greatest amount of Ca^{++} is required, less is required with succinate, and the least with acetate. The increases in growth obtained with the addition of small amounts of Ca^{++} , 10 to 50 γ , with acetate and succinate buffers further indicates that Ca^{++} is the cation responsible for the increased growths. As has been demonstrated by other workers (1), the use of 2 per cent Salts C

gives better growth than 0.5 per cent Salts B (Table II). However, it is to be noted that the addition of 0.2 mg. of Ca^{++} further increases growth with all buffers tested.

DISCUSSION

The data presented indicate that Ca^{++} has an effect on the growth of *L. delbrueckii* other than a sparing action of this cation on the Mg^{++} and Mn^{++} of the basal medium. Spectroscopic analysis¹ of some constituents of the medium shows that some Ca^{++} is present in almost all of the salts used. On the basis of these values the basal media used supplied from 2 to 3 γ of

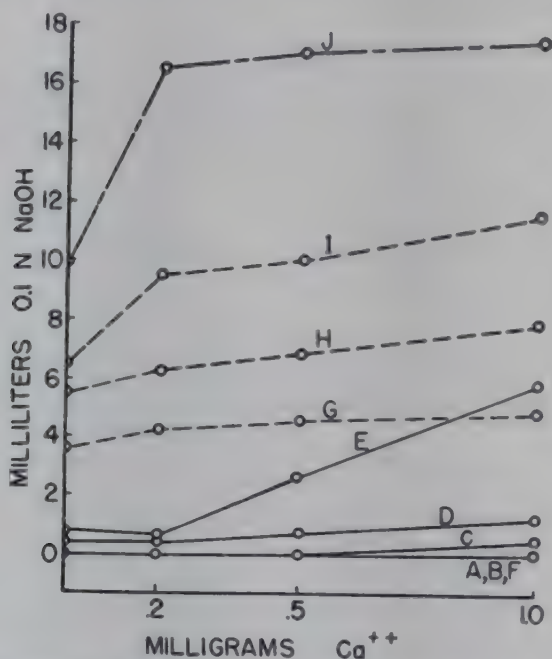


FIG. 2. Calcium studies with limiting leucine and serine. Curves A, B, C, D, E and J obtained with 0, 50, 100, 200, 400, and 2000 γ of DL-serine, respectively; Curves F, G, H, I, and J obtained with 0, 50, 100, 200, and 2000 γ of DL-leucine, respectively. The medium of Henderson and Snell (2) was used.

Ca^{++} per 10 ml. of final medium. With the addition of 10 mg. of Mg^{++} the Ca^{++} level was raised to only 6 to 7 γ . It is evident that these small amounts of Ca^{++} would be significant in a citrate-buffered medium only if a sparing action were exerted by other cations in the medium.

The increased growth obtained with the addition of Ca^{++} to tubes con-

¹ Spectroscopic Ca^{++} determination (micrograms of Ca^{++} per mg.) was as follows: sodium acetate 0.02, sodium citrate $\cdot 2\text{H}_2\text{O}$ 0.005, sodium succinate 0.001, glucose 0.001, KH_2PO_4 0.02, K_2HPO_4 0.003, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0000, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.001, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04, NaCl 0.003. We are grateful to Mr. R. J. Carls of the Physics Department for these calcium determinations.

taining high levels of serine (4 and 8 mg.), the reversal of threonine inhibition of serine utilization by Ca^{++} (Fig. 1), and the relatively small stimulation by Ca^{++} at low growth levels with limiting leucine (Fig. 2) all indicate the importance of Ca^{++} in some phase of serine utilization. Because of this, the addition of Ca^{++} to the basal media for *L. delbrueckii* would be desirable in order to obviate any effects due to Ca^{++} which might be added as sample.

TABLE II

Calcium Studies with Different Buffers

Medium of Stokes *et al.* (5) except glucose and vitamin supplement of Henderson and Snell (2) with 2 γ instead of 10 γ of calcium pantothenate; no threonine, 50 γ of DL-serine.

Ca ⁺⁺ added	0.6 per cent sodium acetate	2 per cent sodium acetate	2 per cent sodium succinate	2 per cent sodium citrate
	Titration, ml. of 0.1 N NaOH			
0.5 % Salts B* per tube				
mg.				
0.0	7.95	5.25	5.85	0
0.01	8.71	6.68	8.00	0
0.025	8.60	8.30	8.78	0
0.05	8.70	9.30	9.02	0
0.10	8.55	9.65	9.60	0
0.20	8.40	9.50	9.70	0
0.50	8.50	9.12	10.30	0
2 % Salts C† per tube				
0.0	7.42	6.50	7.68	1.30
0.10				1.70
0.20	9.42	11.10	11.40	2.65
0.50				5.62
1.00				8.05

* Salts B, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 10 gm., NaCl 0.5 gm., $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 gm., $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.5 gm., water to make 250 ml. of solution.

† Salts C, same as Salts B except 2 gm. instead of 0.5 gm. of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$.

The observation by Bobtelsky and Jordan (6) on the decreased stability of metallic complexes of citrates as the pH is decreased permits an explanation of the increased need for Ca^{++} in threonine-containing media (Fig. 1). Threonine is not essential for *L. delbrueckii* and causes both a lag in the response to serine (4) and a delay in acid production. This delay in acid production keeps the pH of the medium at the initial higher value (6.7 to 6.9) for a greater period of time and, as a result, the stability of the citrate

complex is retained. Without threonine, the shift to the more acid pH occurs sooner and the metal ions are released from the buffer complex. Ca^{++} studies with media of different pH values substantiated such a postulate. In the pH range of 5.5 to 7.05, progressively less Ca^{++} was required to produce a given growth as the pH of the media was decreased. Greater earlier growths were also noted in tubes of lower pH with a given level of Ca^{++} .

The need for different amounts of Ca^{++} with different buffers (Table II) is most readily explained on the basis of the relative cation-binding or complex-forming ability of the buffers. Cannan and Kibrick (7), in a study of the association constants of the alkali earth cations of carboxylic acids, demonstrated that the cation-binding power increases in the order of acetate, succinate, and citrate. The effect of added Ca^{++} parallels the above order in that the least amount of Ca^{++} is required with acetate, intermediate with succinate, and most with citrate. The greater cation-binding power of citrate was also noted in a different way. 10 mg. of Ca^{++} per tube could be added without causing precipitation if citrate were the buffer, whereas only about 1 mg. of Ca^{++} could be kept in solution with acetate or succinate as the buffer. In media for *L. delbrueckii*, either acetate or succinate would appear to be more desirable than citrate as a buffer.

Visual observations made on the growth characteristics of *L. delbrueckii* showed that Ca^{++} not only initiated earlier growth but also changed appreciably the type of growth. In Ca^{++} -containing tubes the growth was characterized by a general turbidity throughout the growth medium. Without Ca^{++} the growth was restricted predominantly to the bottom of the tube. Microscopic examination of cultures disclosed that with Ca^{++} the microorganisms were chiefly in the form of single cells or short chains of cells, whereas, without Ca^{++} the growth was chiefly in the form of long chains. Both of these effects would be important in assay procedures which employ turbidimetric growth measurements after 18 to 24 hour incubation periods. The presence of Ca^{++} in the assay samples could produce earlier growth in the sample tubes than in the standard tubes and thus give high assay values. Also the long chain type of growth could offer difficulties in obtaining homogeneous suspensions for turbidimetric readings.

Limited studies with other microorganisms, *Lactobacillus arabinosus* 17-5, *Leuconostoc mesenteroides* P-60, *Streptococcus faecalis* R, and *Lactobacillus casei* ϵ revealed that only *L. casei* was influenced by the addition of Ca^{++} . This is not surprising, as many of the growth characteristics of *L. casei* and *L. delbrueckii* are similar. The utilization of serine by these two microorganisms is appreciably altered by the presence of threonine (4). In view of this and the data presented on the effect of threonine on the

Ca^{++} requirement of *L. delbrueckii*, it is not unreasonable that the growth of *L. casei* should also be stimulated by the addition of Ca^{++} .

The technical assistance of Janet Dale is gratefully acknowledged.

SUMMARY

1. Data have been presented to show that *Lactobacillus delbrueckii* 9595 requires Ca^{++} as a stimulatory factor for the utilization of serine. The stimulation obtained with added Ca^{++} with other amino acids limiting may be due to an increased utilization of the serine in the basal medium.

2. More Ca^{++} is required by *L. delbrueckii* with threonine in the basal medium. This has been attributed to the antagonistic effect of threonine on serine utilization. High levels of Ca^{++} reverse the toxicity of threonine.

3. With citrate buffer the Ca^{++} requirements are large. Less Ca^{++} is needed with either succinate or acetate buffers. The Ca^{++} requirement depends on the pH of the medium.

4. In tests with *Lactobacillus casei*, *Lactobacillus arabinosus*, *Leuconostoc mesenteroides* P-60, and *Streptococcus faecalis* R, only the growth of *L. casei* was stimulated by the addition of Ca^{++} .

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STEROID EXCRETION IN A CASE OF ADRENOCORTICAL CARCINOMA

IV. Δ^5 -PREGNENETRIOL- $3\beta,16\alpha,20\alpha$ *

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Several years ago we reported (2) the isolation of a new compound from the urine of a boy with an adrenocortical tumor. This substance which was provisionally designated as Compound A was obtained from the non-ketonic fraction that precipitated with digitonin. It has the composition $C_{27}H_{44}O_3$ and was characterized as a triol with three reactive hydroxyl groups, since it readily formed a triacetate $C_{27}H_{40}O_6$ upon acetylation at room temperature. Since that time, larger amounts (47 mg.) of this compound have been isolated which have permitted its identification as Δ^5 -pregnenetriol- $3\beta,16\alpha,20\alpha$ (VIII).

If the analytical data are interpreted on the basis of the tetracyclic structure of the steroids, they indicate the presence of one olefinic double bond. This was verified by the catalytic reduction of the triacetate (VII), which furnished a saturated ester (VI) which contained 2 additional hydrogen atoms. The reaction was accompanied by a large increase in molecular rotation¹ ($+197^\circ$ in alcohol). Such a shift is in harmony with the assumption that a double bond in the 5-6 position was reduced to a 5- α -steroid (Table I). Moreover, as has been pointed out previously (7), an increase of this magnitude has not been observed with other monoolefinic steroids.²

* Presented in part at the fortieth annual meeting of the American Society of Biological Chemists in Detroit (1). This investigation has been supported by grants from the Bourne Fund and from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

¹ Up to the present report we have used the term as defined by Bernstein, Kauzmann, and Wallis (3): $[M]_D = [\alpha]_D \times \text{molecular weight}$. Since most of the recent literature uses $[M]_D = [\alpha]_D \times \text{molecular weight} \times 10^{-2}$, we are now conforming to this practice. In most computations specific rotations or their averages were rounded off to full degrees.

² Barton in his very extended surveys of the rotation of unsaturated steroids recently assigned a similar value ($+200^\circ$) to the reduction of Δ^6 -steroids (8). This estimate, however, was based on examples which are likely to be subject to disturbances from substituents or double bonds adjacent to the 6-7 double bond. One of these compounds, Δ^6 -cholestenediol- $3\beta,5$ has recently been shown to be far less levorotatory than had hitherto been assumed (9). ($\Delta[M]_D^{\text{sat.}} - \Delta^6 = 77^\circ - (-64^\circ) = +141^\circ$ for this compound (9, 10).)

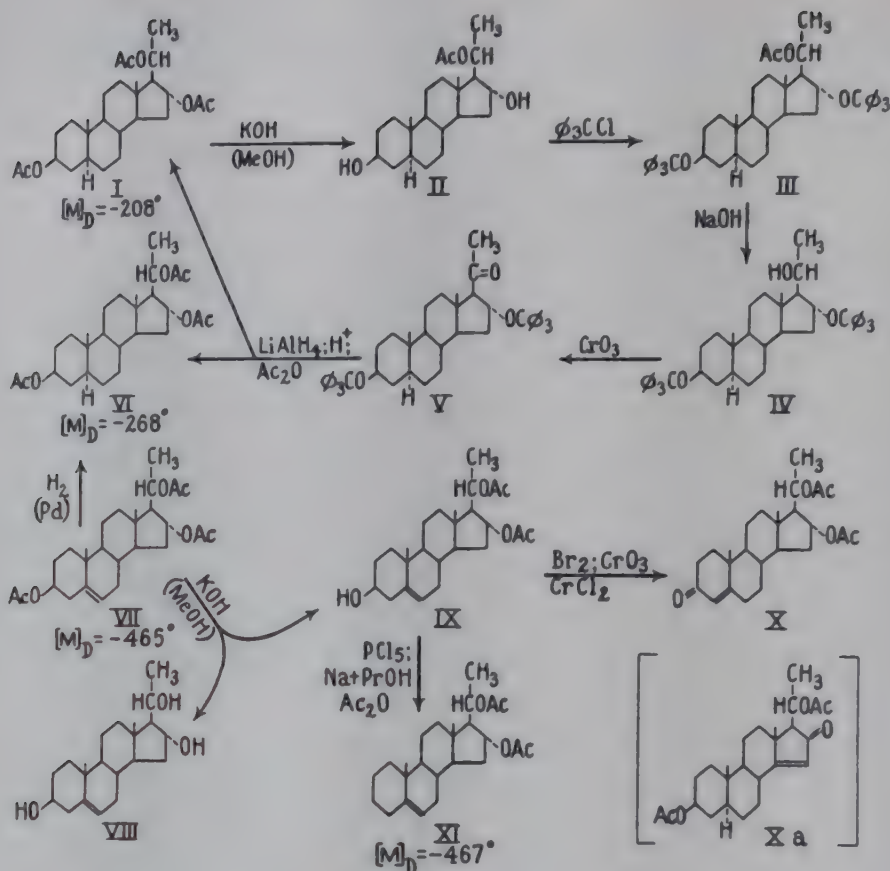


TABLE I

Differences of Molecular Rotations between 3 β -Acetoxy-5-allosteroids and 3 β -Acetoxy- Δ^5 -steroids in Alcohol*

The figures in parentheses refer to the bibliography.

Saturated compound	$[M]_D$	Δ^5 compound, $[M]_D$	$\Delta[M]_D^{5\text{-allo}} - \Delta^5$
	degrees	degrees	degrees
3 β -Acetoxyallopregnanone-20	+288 (5)	+79 (6)	+209
3 β ,20 α -Diacetoxyallopregnane	+8†	-181†	+189
3 β ,20 β -Diacetoxyallopregnane	+125‡	-93‡	+218
3 β -Acetoxyandrostanone-17	+249 (4)	+20 (4)	+229
3 β ,16 α ,17 β -Triacetoxyandrostane	-191 (7)	-441 (7)	+250
3 β -Acetoxycholestane	+82 (4)	-137 (4)	+219

* Barton (4) was the first to point out that $\Delta[M]_D^{5\text{-allo}} - \Delta^5$ is significantly less in alcohol than in chloroform.

† See the experimental part.

‡ Unpublished data from this laboratory.

This location of the double bond would eliminate C-6 as a possible site of attachment of one of the hydroxyl groups. One of these was suspected to

be located in the 3β position, since Compound A precipitated with digitonin, and a second in the 20α position³ in view of the source of the isolated substance. If these conjectures were correct, the third hydroxyl was presumably not attached to carbon atoms 2, 4, 17, or 21, since Compound A proved to be stable towards periodic acid. Positions 5, 8, 9, 11β , and 14 seemed to be excluded by the fact that all three hydroxyl groups can be readily acetylated. The reduced triacetate (VI) is far more levorotatory than the diacetate of allopregnanediol- $3\beta, 20\alpha$ ($[M]_D = +8^\circ$). Therefore, if our surmises about the locations of two of the acetoxy groups are correct, the third makes a very large negative contribution (-276°) to the molecular rotation. Survey of the literature, however, disclosed no such effect of any of the positions still to be considered, with the possible exception⁴ of 11α which shows very large variations with changes of the substituents at C-17. Table II gives the current status of this information; when this investigation was started (1946), no data were available concerning the two isomers at C-15 and one each at C-1, C-16 (16α), C-18, and C-19.

Since the allopregnanetriol- $3, 16, 20$ of the urine of pregnant mares (Marrian's triol) was reported as levorotatory (48, 49), the 16 position warranted further investigation. Its triacetate (I) was found to have a

³ The terms α and β are used as defined previously (11). Specifically, substituents at C-7 are named in accordance with Fieser *et al.* (12), at C-11 and C-12 with Gallagher and Long (13) and Sorkin and Reichstein (14), and at C-17 with Fieser and Fieser (15). The identity of the configurations of the hydroxyl groups at C-20 in urinary pregnanediol and in urinary allopregnanediol- $3\beta, 20$, which is basic to all our deductions at this asymmetric center, has been rigorously proved in this laboratory by a direct comparison of two preparations of Δ^4 -pregnenol-20-one-3 acetate that were obtained from pregnanediol by the method of Butenandt and Schmidt (16) and from urinary Δ^5 -pregnenediol- $3\beta, 20$ (17, 2) by oxidation of the 20-monoacetate. The same conclusion can be reached by comparing the results of Sarett (18) with those of Klyne and Barton (19) who determined the rotational shift of 20-hydroxy compounds upon acetylation. These investigations established further that the definition of the 20α configuration used here (11) is in accord with that proposed by Fieser and Fieser (15). We are greatly indebted to Dr. Klyne who very kindly sent us a copy of their manuscript before publication.

⁴ 7α -Acetoxy compounds are also characterized by a fairly large negative contribution of the acetoxy group, but can be excluded since the 7-acyloxy group in Δ^5 -steroids undergoes hydrogenolysis on catalytic reduction (20). Even if we were able to avoid this, the shift calculated for the reduction of the double bond ($+773^\circ$; chloroform) is wholly anomalous and incompatible with our measurements on the reduction of Compound A triacetate. In this calculation the usual assumption was made that the levorotatory Δ^5 -cholestenediol- $3\beta, 7$ diacetate ($[M]_D = -856^\circ$ (21, 22, 9)) has the same configuration at C-7 as the levorotatory cholestanediol- $3\beta, 7$ diacetate ($[M]_D = -83^\circ$ (23)). If the dextrorotatory Δ^5 -cholestenediol- $3\beta, 7$ diacetate ($[M]_D = +258^\circ$ (20, 22)) were stereochemically related to the levorotatory cholestanediol- $3\beta, 7$ diacetate, the calculated value for the reduction of the Δ^5 double bond would be even more anomalous (-341°).

TABLE II

*Effect of Acetoxy Groups on Molecular Rotations**

The figures in parentheses refer to the bibliography.

Position	Configuration	Substituted compound	$[M]_D^{OAC}$	$[M]_D^H$	$\Delta[M]_D^{OAC-H}$	Sol- vent†
			<i>degrees</i>	<i>degrees</i>	<i>degrees</i>	
1	γ γ	1-Acetoxycholestane	+41 (25)	+91 (26)	-50	Ch.
7	α	3 β ,7 α -Diacetoxycholestane	-83 (23)	+60 (26)	-143	"
	β	3 β ,7 β -Diacetoxycholestane	+268 (23)	+60 (26)	+208	"
11	α	3 α ,11 α -Diacetoxypregnanone-20	+310 (27)	+301 (28)	+9	"
		Methyl 3 β ,11 α -diacetoxyetio- cholanate	+113 (29)	+184 (30)	-71	"
		11 α -Acetoxyetiocholanol-3 α - one-17	+87 (31)	+317 (31)	-230	"
12	α	Methyl 3 α ,12 α -diacetoxycho- lanate	+463 (32)	+207 (33, 13)	+256	Ac.
		Methyl 3 β ,12 α -diacetoxy- Δ^6 - cholenate‡	+34 (34)	-82 (35)§	+116	Ch.
		Methyl 3-keto-12 α -acetoxy- Δ^4 - etiocholenate	+750 (36)	+479 (37)	+271	Ac.
		12 α ,21-Diacetoxy- Δ^4 -pregnene- dione-3,20	+852 (38)	+611 (39)	+241	"
	β	Methyl 3 α ,12 β -diacetoxycho- lanate	+279 (32)	+207 (33, 13)	+72	"
15	α β					
16	α	3 β ,16 α ,20 α -Triacetoxyallopreg- nane	-268	+8	-276	Al.
		3 β ,16 α ,20 α -Triacetoxxy- Δ^6 - pregnene	-465	-181	-284	"
		3 β ,16 α ,20 β -Triacetoxyallo- pregnane	-208 (11)	+125¶	-333	"
		3 β ,16 α ,20 β -Triacetoxyallo- pregnane	-208 (40)	+89 (19)	-297	Ch.
		3 β ,16 α ,17 β -Triacetoxxy- Δ^6 - androstene (41)	-441 (7)	-211 (42)	-230	Al.
	β	3 β ,16 β ,20 α -Triacetoxyallo- pregnane	+116 (11)	+8	+108	"
		3 β ,16 β ,20 β -Triacetoxyallo- pregnane	+213 (11)	+125¶	+88	"
		Methyl 3 β ,16 β -diacetoxyetio- cholanate	+135 (43)	+184 (30)	-49	Ch.
18						
19		Methyl 3 β ,19-diacetoxyetioallo- cholanate‡	+148 (44)	+139 (45, 46)	+9	Ch.
		Methyl 19-acetoxyetioallocho- lanate‡	+158 (44)	+156 (47, 46)	+2	"

Table II—*Concluded*

* Although it is not claimed that these data represent a complete survey of the literature, it should be noted that rotations are cited only if both members of the pair under comparison were measured in the same solvent. (No distinction is made between 95 per cent and absolute ethanol.) Furthermore, compounds with cis fusion, of Rings A and B or C and D are not cited if the acetoxy group is attached to such a ring, or to the angular methyl group between two cis-linked rings. A very condensed survey of $\Delta[M]_D^{OAc-H}$ has recently appeared (24).

† Ch. = chloroform, Ac. = acetone, Al. = alcohol.

‡ Some reservation about the identity of this product has been expressed by the authors.

§ It is not quite clear from the text whether the rotation $[\alpha]_D = -18.7^\circ$ measured was for the acetate methyl ester (as is assumed here) or for the free acid.

|| See the experimental part.

¶ Unpublished data from this laboratory.

TABLE III

Differences of Molecular Rotations between 3 β -Acetoxy-5-allosteroids and 3 α -Acetoxy-5-allosteroids in Alcohol

3 β compound	$[M]_D$	3 α compound, $[M]_D$	$\Delta[M]_D^{3\beta-3\alpha}$
	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
3 β -Acetoxyandrostanone-17.....	+249 (4)	+289 (53)	-40
3 β ,20 α -Diacetoxyallopregnane.....	+8*	+65*	-57
3 β -Acetoxyallopregnanone-20.....	+288 (5)	+341 (5)	-53

* See the experimental part.

molecular rotation of -208° (11), only 60° more positive than our reduced triacetate. Since Marrian's triacetate was considered to be a 3 α -steroid (50-52), epimerism at C-3 (Table III) seemed to account satisfactorily for the observed difference in rotation. However, our attempts to convert the triol of mare urine into its 3 β isomer were unsuccessful and revealed the fact that Marrian's triol is not a 3 α compound but has the structure of allopregnanetriol-3 β ,16 α ,20 β (11). While this finding eliminated epimerism at C-3 as the basis of the structural difference between these two triacetates, it suggested at the same time that our reduced compound might be the 20 α epimer of the mare triol since this would again, within acceptable limits, account for the observed difference in molecular rotation (Table IV). We, therefore, considered allopregnanetriol-3 β ,16 α ,20 α as the most likely structure for Dihydrocompound A and have verified this hypothesis by the synthesis of this substance from the 20-epimeric triol of the urine of pregnant mares.

Treatment of the triacetate from mare urine (I) with very dilute meth-

anolic potassium hydroxide at room temperature for prolonged periods leads to the formation of a monoacetate (51) which is esterified in position 20 (II) (11, 52). In order to preserve the configurations of the free hydroxyl groups at C-3 and C-16, triphenylmethyl ethers were formed, since these groups are stable towards alkali but can be readily hydrolyzed with acid. In the steroid field trityl ethers have been prepared by Josephson (54), by Riegel *et al.* (55), and by others (56, 57). Treatment of monoacetate II with triphenylmethyl chloride furnished compound III in good yield. Comparison of its absorption spectrum with those of other trityl ethers (Fig. 1) as well as the carbon and hydrogen analysis showed that both hydroxyl groups had reacted. The acetoxy group at C-20 was selectively hydrolyzed with sodium hydroxide in aqueous ethanol. The reaction product (IV) was not readily differentiated from the starting compound III, since both have the same melting points and show no depression on admixture. The non-identity of these substances, however,

TABLE IV
Differences of Molecular Rotations between 20 α -Acetoxysteroids and 20 β -Acetoxysteroids in Alcohol

20 α compound	$[M]_D$	20 β compound, $[M]_D$	$\Delta[M]_D^{20\alpha - 20\beta}$
	degrees	degrees	degrees
3 β , 16 β , 20 α -Triacetoxyallopregnane....	+116 (11)	+213 (11)	-97
3 β , 20 α -Diacetoxyallopregnane.....	+8*	+125†	-117
3 β , 20 α -Diacetoxy- Δ^5 -pregnene.....	-181*	-93†	-88

* See the experimental part.

† Unpublished data from this laboratory.

was established by a comparison of the infra-red spectra, since compound IV in contrast to substance III shows a hydroxyl but no carbonyl or ester peak (8.08 μ). In the finger-print region from 8.2 to 11.3 μ numerous differences also exist, but from 12 to 15 μ the two curves are virtually identical. In this range (Fig. 2) they are easily distinguished from tritanol which forms from these ethers with great ease on any exposure to acid. This spectrographic behavior simplified the analysis of mixtures of tritanol with trityl ethers of this type and permitted us to explore reaction conditions with trial runs on a rather small scale (about 3 mg.). Detritylation occurred to a significant extent when compound IV was oxidized with chromium trioxide, although the reaction medium was acetone rather than the customary acetic acid. The main product in this reaction, nevertheless, was the desired 3 β , 16 α -ditritoxyallopregnanone-20 (V), since it had the composition of a ditrityl ether and showed an absorption maximum at 5.86 μ , a characteristic of 20-ketosteroids (58).

The only known procedure for the reduction of 20-ketosteroids which furnishes 20 α compounds in satisfactory yields was treatment with sodium and ethanol (28). When this reaction was applied to ketone V, extensive formation of free tritanol was observed. Since it seemed very unlikely that an ether linkage had been solvolyzed in an alkaline medium, the reaction was considered to be an elimination of tritanol facilitated by the un-

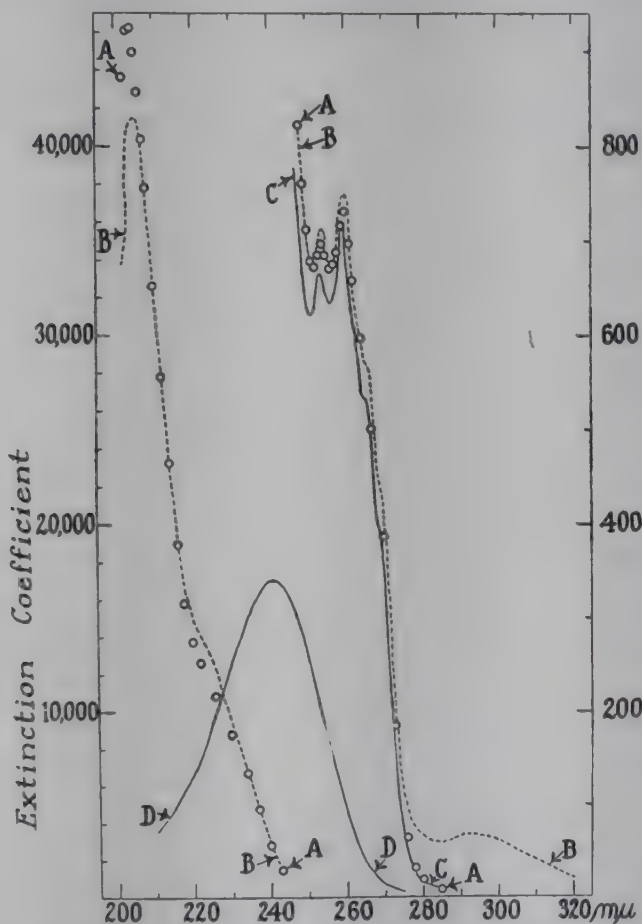


FIG. 1. Ultraviolet absorption spectra. Curve A (O), 3 β ,16 α -ditritoxy-20 β -acetoxyallopregnane (III); Curve B (broken line), 3 β -tritoxysterone-17; Curve C (solid line), tritoxysterone; Curve D (solid line), Δ^4 -pregnenediol-16 α ,20 α -one-3 diacetate (X). Curves A to C are plotted as ϵ/N (N = number of tritoxy groups per molecule) at and above 247 $m\mu$ with respect to the ordinate at the right and below that with respect to the ordinate at the left. Curve D is plotted as ϵ with reference to the left ordinate.

saturation of the β -carbon atom (C-20).⁵ Searching for a procedure which would permit the retention of the substituent at C-16, we found that the reduction of Δ^5 -pregnenol-3 β -one-20 acetate with lithium aluminum hydride (60) yields a mixture of the two epimeric forms of Δ^5 -pregnenediol-3 β ,20

⁵ An analogous explanation was preferred by Kuhn and Winterstein (59) for the alkaline cleavage of picrocrocin into glucose and safranal.

which contains appreciable amounts of the α form, and analogous results in the reduction of another 20-ketosteroid have been reported since then by others (61). Although the use of Grignard's reagent, which in general parallels the action of this hydride, has been stated to effect cleavage of trityl ethers (62), no such reaction was observed when ketone V was treated with lithium aluminum hydride. The reaction product was hydrolyzed with acid and after acetylation fractionated by chromatography. Again, two isomers were obtained. One of these was identical with the starting material (I), demonstrating (as was to be anticipated) that the hydrolysis of the tritoxyl groups proceeds without inversion. The second triacetate

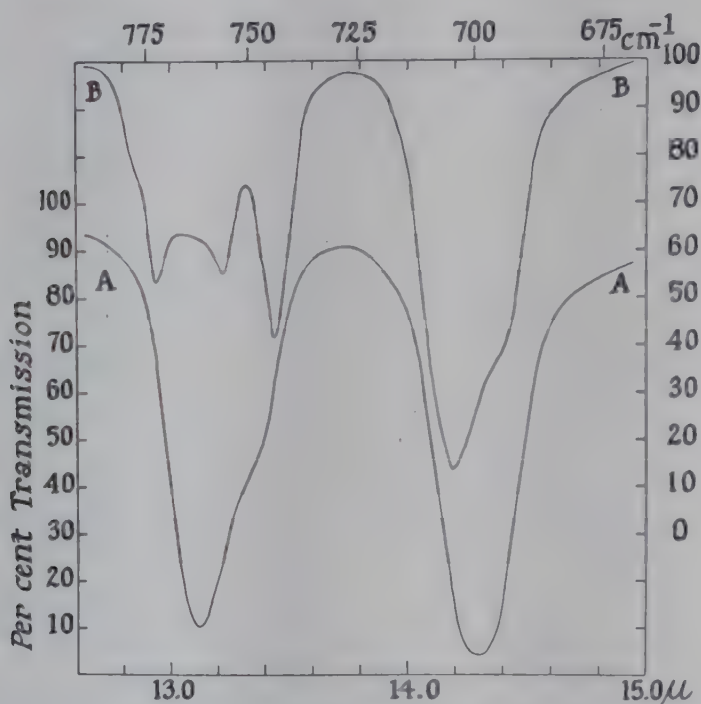


FIG. 2. Infra-red transmission spectra. Curve A, tritanol; Curve B, $3\beta,16\alpha$ -ditritoxy- 20β -acetoxyallopregnane (III). Curve A is drawn with reference to the left, Curve B to the right ordinate.

was found to be identical with the reduction product of the isolated triacetate. Comparison included determinations of the mixed melting points of the free triols as well as of the triacetates and measurements of their rotations and infra-red spectra (Fig. 3, Curves A and B). Since the synthetic route used appears to be free of ambiguity, the reduced triol must be the 20-epimer of the triol from mare urine and consequently is to be designated as allopregnanetriol- $3\beta,16\alpha,20\alpha$.

The rotational changes which accompanied the reduction of triacetate VII were regarded as a strong indication that the double bond occupied the 5-6 position. This was fully corroborated by oxidation. Since treatment

of Marrian's triol with chromic acid even under mild conditions produced far reaching changes (48, 52), we subjected a partially acetylated derivative of Compound A rather than the free triol to this reaction. This derivative (IX) had the composition of a diacetate and was obtained together with the free triol by the partial methanolysis of the triacetate VII. The diacetate was brominated, oxidized with chromic acid, and debrominated by the excellent procedure of Julian, Cole, Magnani, and Meyer

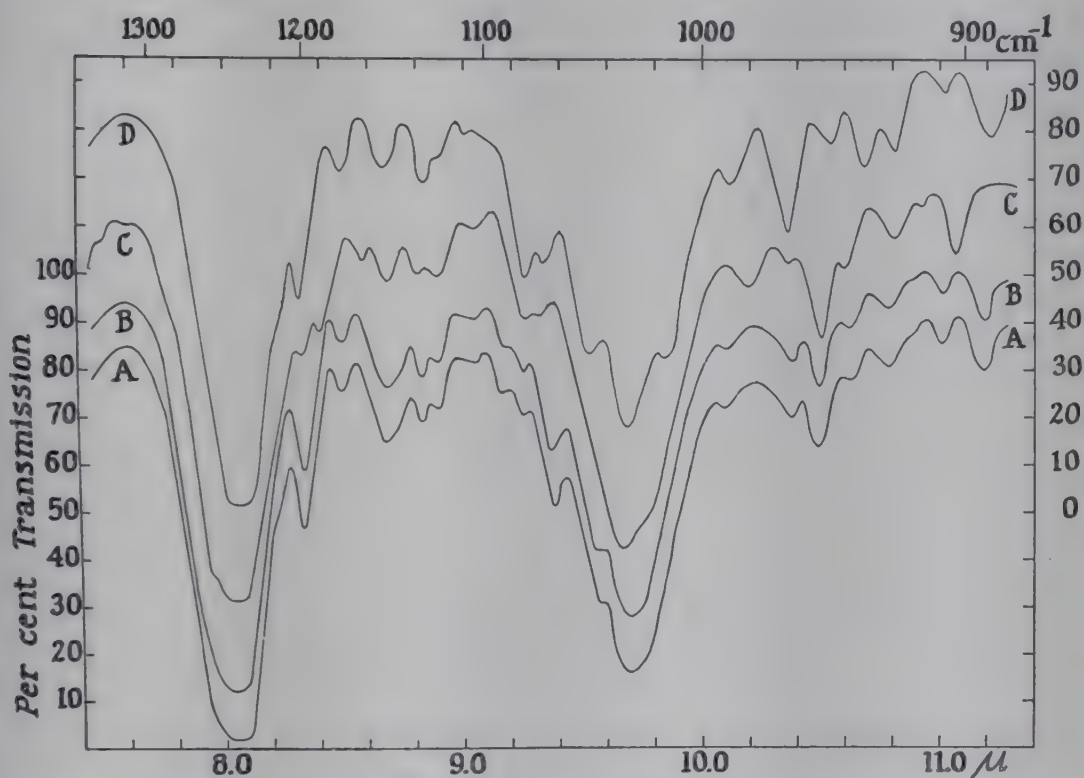


FIG. 3. Infra-red transmission spectra. Curve A, $3\beta, 16\alpha, 20\alpha$ -triacetoxyallopregnane (VI) from compound VII; Curve B, compound VI from compound V; Curve C, $3\beta, 16\alpha, 20\alpha$ -triacetoxy- Δ^5 -pregnene (VII); Curve D, $3\beta, 16\alpha, 20\beta$ -triacetoxyallopregnane (I) from mare urine. Curves B and C are displaced by +10 and +30, respectively, from Curve A which is drawn with reference to the ordinate at the left. The ordinate for Curve D is given on the right.

(63). The reaction product (X) was an α - β -unsaturated ketone, since it exhibited an intense absorption maximum at $240.5 \text{ m}\mu$ (Fig. 1). An α - β -unsaturated ketone may be expected to form from the diacetate of an allopregnenetriol-3, 16, 20 either by conjugation of the original double bond with the newly formed ketone group or far less likely by the elimination of acetic acid if the acetoxy group is β to the carbonyl group. The composition of the reaction product, $\text{C}_{25}\text{H}_{36}\text{O}_5$, showed that both acetoxy groups of the starting compound are still present and therefore eliminates

the second possibility. Consequently, the α - β -unsaturated ketone must have one of these three structures: *viz.*, (1) Δ^1 -allopregnenediol-16 α ,20 α -one-3 diacetate, (2) Δ^4 -pregnenediol-16 α ,20 α -one-3 diacetate (X), (3) Δ^{14} -allopregnenediol-3 β ,20 α -one-16 diacetate (Xa). Since Δ^1 -3-ketosteroids in alcohol have an absorption maximum at (or below) 230 m μ (64), such a structure need not be considered further. Structure Xa also seems to be inadmissible on spectrographic grounds, since a 3-methoxy- $\Delta^{1,3,5-10,14}$ -estratetraenone-16 shows an absorption peak at 227 m μ which was ascribed to the α - β -unsaturated keto group (65). While the benzenoid ring in this compound undoubtedly makes a significant contribution to the absorption in this region, it is not to be expected that the superposition would cause a major shift of the wave-length of maximal extinction. Moreover, if structure Xa were correct for the oxidation product, the reductive removal of the free hydroxyl group in its precursor, the triol diacetate, should yield Δ^{14} -allopregnenediol-3 β ,20 α diacetate. However, the molecular rotation of the resulting desoxy compound differs from that of allopregnanediol-3 β ,20 α diacetate by -475° , which seems incompatible with the change observed upon the introduction of a double bond into the 14-15 position ($+28^\circ$ (24)) or into any other that may conceivably migrate there upon oxidation. On the other hand if the α - β -unsaturated ketone has the alternative structure X, the location of the absorption maximum should be very close to the observed value. Such a compound must be formed from the 16,20-diacetate (IX) of the pregnenetriol. Substitution of its free hydroxyl group by hydrogen would yield a pregnenediol-16,20 diacetate (XI) differing from the triacetate VII in the absence of the 3 β -acetoxy group. The molecular rotation calculated for such a structure (-484°)⁶ is in satisfactory agreement with our observation (-467°). We conclude, therefore, that the α - β -unsaturated ketone has structure X and that it is formed by the oxidation of either a Δ^4 - or Δ^5 -stenol-3. Since the reduction of a 4-5 in contrast to a 5-6 double bond produces a levorotatory shift, and since Compound A does not give the Rosenheim reaction (66), the double bond in the isolated triol cannot occupy the 4-5 position. Accordingly we regard the structure of Δ^5 -pregnenetriol-3 β ,16 α ,20 α as the only one which is in harmony with all our observations on Compound A.

The assignment of configurations of the 16-hydroxy groups of the allopregnanetriols-3 β ,16,20 was based on the reactions of the sapogenins and their conversion products as well as on the results of the alkali-catalyzed

⁶ Estimate based on rotation of compound VII and the difference of the molecular rotations (-19° ; chloroform) between Δ^5 -cholestene (26) and cholesteryl acetate (26).

methanolysis of the allopregnanetriol triacetates (11). Under standardized conditions the triacetates of allopregnanetriol-3 β ,16 β ,20 α and of allopregnanetriol-3 β ,16 β ,20 β gave the 16,20-diacetates as the main reaction products, while allopregnanetriol-3 β ,16 α ,20 β triacetate furnished the 20-monoacetate in good yield. Since the three compounds under consideration are stereoisomers, it seemed more plausible to ascribe the difference in reaction rates to steric hindrance than to effects transmitted through the carbon chains. Since the side chain at C-17 is close to a 16 β -acetoxy group, the slower reaction of this substituent seemed adequately explained by its orientation. On this basis we expected the formation of a 20-monoacetate when a 16 α ,20 α compound, triacetate VII, was treated in the same manner. The isolation of 51 per cent of diacetate IX and 26 per cent of free triol (VIII) does not conform to this simple pattern. As it is not to be expected that the presence of a double bond in the 5-6 position would influence the solvolysis rate of an acetoxy group as distant as one at C-16, an explanation for the different behavior of the two triacetates (I and VII) was to be sought in the configuration of C-20. While it is possible, of course, to arrange the substituents of this asymmetric center regardless of their configurations in such a manner that they are far removed from a 16 α -acetoxy group, such an orientation apparently is not favored in compound VII, since the prevalence of the 16,20-diacetate among the reaction products indicates an inhibitory effect. The substituent at C-20 which can most effectively shield the carboxy carbon of a 16 α -acetoxy group is the 20-acetoxy group, if it is directed towards the ester linkage at C-16. It appears, therefore, that the bond C-20 $\rightarrow$ O in compound VII extends preferably in a somewhat more forward direction than that of the linkage C-13 to C-17. The presence of relatively large amounts of triol VIII in the absence of larger amounts of monoacetates demonstrates the interdependence of the solvolysis reactions at C-16 and C-20. This again suggests the close approach of the two ester groups in compound VII, since the solvolysis of one of these substituents should reduce its size and therefore abolish its inhibitory effect. The behavior of triacetate I indicates that the preferred orientation of its 20-acetoxy group is not towards the 16 α -acetoxy group. This difference would be difficult to explain if the space at the side of the angular methyl group and of C-12 were equally available for either the hydrogen atom or the methyl group attached to C-20. Inspection of molecular models leaves little doubt, however, that the relatively crowded space near the angular methyl group can accommodate a hydrogen atom much more readily than the methyl group. It may be reasoned then that the close approach of the two ester groups is facilitated by that configuration which places the hydrogen atom next to the angular methyl group if

the bond C-20 to C-21 extends somewhat to the rear of the general direction of the C-16 to C-17 linkage. This, therefore, should be the preferred location of the substituents at C-20 in compound VII under the conditions of the methanolysis reaction. This arrangement implies a configuration of the 20 α -hydroxy group which is identical with that derived by Fieser and Fieser (15). Furthermore, the projections obtained in the x-ray analysis of cholesteryl iodide (67) (particularly with crystal modification B) picture the bonds C-20 to C-21 and C-20 to C-22 in about the positions suggested for the 20-methyl and 20 α -acetoxy groups respectively of compound VII. It is probable, although by no means certain, that the configuration of the isohexyl group of cholesterol agrees with that of the 20-hydroxy group of pregnanediol-3 α ,20 α .⁷ Diosgenin has been converted to cholesterol (69) by reactions which are expected to retain the configuration at C-20,⁸ and its reduction product tigogenin has been converted to an allopregnanetriol-3 β ,16,20 (71) which has been shown to have the 20 α configuration (11). This conversion involves a persulfuric acid oxidation which seems to proceed without inversion (72). The agreement of our deductions with these findings lends support to the explanation given for the different behavior of the triacetates I and VII in the solvolysis reaction. Nevertheless, a more direct experimental test for these concepts seems highly desirable and will be attempted in this laboratory.

The yield of Δ^5 -pregnenetriol-3 β ,16 α ,20 α amounted to 6 mg. per liter of urine. It seems most improbable that this compound would have escaped detection until this time if its concentration in normal urine were of the same order of magnitude as that encountered by us. Accordingly we conclude that the isolated compound is derived either wholly or to a large extent from a substance or substances formed by the adrenal tumor and its metastases. This demonstrates, we believe for the first time, that adrenal tissue can give rise to steroids of the C₂₁ series that are oxygenated in position 16. The process presumably cannot be dismissed as a pathological curiosity, since a related triol with the same spatial orientation of the 16-hydroxyl group has been found consistently in the urine of pregnant mares. There is, however, as yet insufficient information to decide whether the 16-oxygen atom is introduced into the precursor of pregnenetriol during its formation by the adrenal or subsequently during the course of its metabolism in other organs. If 16-oxygenated steroids should prove to be native to the adrenal cortex, the effect of this type of substitution on biological activity may be of considerable interest.

⁷ Additional evidence bearing on this point has been provided by Wieland and Miescher in a paper (68) received after the completion of this manuscript.

⁸ The first step involves a Clemmensen reduction of the ketal at C-22 which could lead to inversion at C-20. No rearrangement at C-20, however, has been described in other reactions which exposed sapogenins to mineral acids (70).

EXPERIMENTAL⁹

Δ^5 -Pregnenetriol-3 β ,16 α ,20 α (VIII)—The isolation of Δ^5 -pregnenetriol-3 β ,16 α ,20 α triacetate (VII) from the acetylated non-ketonic fraction that precipitated with digitonin, its analysis, and hydrolysis have been described previously (acetate of Compound A (2)). Since this time we have isolated this triacetate with a slightly sharper melting point (178.5–180°) from the remaining extracts (7) of the patient's urine.

Rotation of Triacetate— $[\alpha]_D^{25} = -101^\circ$ ($c = 0.8$)

The infra-red spectrum is given in Fig. 3, Curve C.

The yield of compound VIII from two of the three aliquots of the urine extracts under investigation amounted to 6 mg. per liter of urine. A solution of the triol in 80 per cent ethanol formed a flocculent precipitate with digitonin within 5 minutes. A solution of 0.9 mg. of Δ^5 -pregnenetriol-3 β ,16 α ,20 α in 0.4 cc. of methanol was treated with 0.1 cc. of a 1 per cent aqueous solution of periodic acid and kept at room temperature (26°) for 20 hours. The neutral ether-soluble material was once recrystallized and identified as starting material by its melting point and mixed melting point. A solution of the triol in 90 per cent trichloroacetic acid (Rosenheim reaction) remained colorless for more than 2 hours.

Allopregnanetriol-3 β ,16 α ,20 α Triacetate (VI) by Reduction of Δ^5 -Pregnenetriol-3 β ,16 α ,20 α Triacetate (VII)—A solution of 24.6 mg. of pregnenetriol triacetate in 9 cc. of 95 per cent ethanol was added to a suspension of reduced palladium-calcium carbonate catalyst (218 mg., 1 per cent (73)) in 4 cc. of alcohol and shaken in an atmosphere of hydrogen at 27° at normal pressure. After 16 minutes 1 molar equivalent of gas was taken up and the reaction ceased. The reaction product was separated from the catalyst by centrifugation, taken to dryness, dissolved in ether, and washed with dilute hydrochloric acid (to remove remaining traces of calcium car-

⁹ All melting points reported are corrected. Rotations were determined in 95 per cent ethanol. Samples were dried for analysis and rotation at 80° *in vacuo* except when noted otherwise. Infra-red transmission measurements were made on carbon disulfide solutions at a concentration of 1 mg. per 0.1 cc. with a cell spacer of 0.8 mm. When the measurements recorded in this and the previous paper (11) were made, the resolution given by the instrument (Perkin-Elmer spectrometer with d.c. amplification) was less than the maximum attainable. The ultraviolet absorption measurements were made with a Beckman spectrophotometer in 1 cm. cells on solutions in 95 per cent ethanol.

The experiments on the reduction of Δ^5 -pregnenol-3 β -one-20 acetate with lithium aluminum hydride, on the preparation of Δ^5 -pregnenediol-3 β ,20 α 20-monoacetate by the partial methanolysis of the diacetate, and on the comparison of its oxidation product with Butenandt's Δ^4 -pregnenol-20 α -one-3 acetate, which are mentioned in the theoretical section, were carried out in collaboration with Dr. Margaret Daus and will be reported in detail at a later date.

bonate) and with water. The dry residue of the ether phase was recrystallized from methanol. 22.0 mg. of needle-shaped crystals were obtained which melted at 177–179°.

Analysis— $C_{27}H_{42}O_6$. Calculated, C 70.10, H 9.15; found, C 70.11, H 9.16

Rotation— $[\alpha]_D^{20} = -58^\circ$ ($c = 0.6$)

A mixture with the starting compound (VII) melted at 174–177°. In spite of the slight depression, non-identity of the two products follows conclusively from a comparison of their rotations and of their infra-red spectra (Fig. 3). The reduced triacetate in concentrated sulfuric acid gave a yellow solution with blue-green fluorescence, while the starting compound showed a brown solution with green fluorescence.

Allopregnanetriol-3 β ,16 α ,20 α —A solution of 12.3 mg. of triacetate VI (obtained by reduction of compound VII) in 4.2 cc. of 95 per cent ethanol was mixed with 0.8 cc. of 4 per cent aqueous sodium hydroxide and heated under a reflux for 100 minutes. The mixture was distributed between ether and water. The washed ether phase yielded 8.0 mg. of crystalline residue. This was dissolved in a mixture of 0.08 cc. of methanol and 0.4 cc. of benzene. The solution was boiled until crystals started to form, treated with 0.04 cc. of petroleum ether, and chilled. The triol, precipitated in this manner, no longer showed any tendency to separate from aqueous ethanol as a gel. Recrystallization from this solvent yielded elongated hexagonal plates melting at 251–253°. The melt was colorless.

Analysis—Sample dried at 110° *in vacuo*

$C_{21}H_{36}O_3$. Calculated, C 74.95, H 10.78; found, C 75.29, H 11.05

Treatment of the triol with pyridine and acetic anhydride for 17 hours at room temperature furnished the starting compound (triacetate VI) which melted after recrystallization at 176.5–178.5°.

Partial Solvolysis of Δ^5 -Pregnenetriol-3 β ,16 α ,20 α Triacetate (VII); Δ^5 -Pregnenetriol-3 β ,16 α ,20 α 16,20-Diacetate (IX)—37.6 mg. of the triacetate VII, 0.0816 mm of sodium hydroxide, and 13.2 cc. of methanol (resulting molarity of both solutes 0.00618) were kept at 20° for 48 hours, after which 100 cc. of ether and 70 cc. of water were added. The ether phase was washed with water and yielded 30.5 mg. of residue (average molecular weight 373). (When the methanolysis was repeated with triacetate from the reacylated mother liquors, an average molecular weight of 388 was obtained, which seems in better accord with the yields and nature of the products isolated.) This material was separated by repeated treatments with small volumes of acetone into a soluble and insoluble fraction. (When the removal of the insoluble material was completed, 15 mg. of the diacetate could readily be dissolved in 0.5 cc. of acetone at room temperature.)

Both fractions were recrystallized from 95 per cent ethanol. The material which was only sparingly soluble in acetone gave 5.5 mg. of needle-shaped crystals which melted at 245–248° with decomposition and showed no depression of the melting point when mixed with Δ^5 -pregnenetriol-3 β ,16 α ,-20 α (VIII). The mother liquors gave 1.6 mg. of a somewhat less pure product. The acetone-soluble fraction yielded 17.5 mg. of fine needles melting at 180.5–184.5°. The analytical specimen of the diacetate IX melted at 185.5–187.5°.

Analysis—Sample dried at 110° *in vacuo*

$C_{25}H_{38}O_5$. Calculated, C 71.74, H 9.15; found, C 71.92, H 9.16

Oxidation of Δ^5 -Pregnenetriol-3 β ,16 α ,20 α 16,20-Diacetate (IX); Δ^4 -Pregnenediol-16 α ,20 α -one-3 Diacetate (X)—A solution of 8.4 mg. of diacetate IX in 0.5 cc. of glacial acetic acid was treated with 0.020 mm of bromine in 0.22 cc. of the same solvent and then with 0.020 mm of chromium trioxide in 0.2 cc. of 90 per cent acetic acid. After the mixture had stood at 25° for 2 hours, 0.3 cc. of methanol was added. After 20 minutes the solution was diluted with 2.4 cc. of acetone. The air in the reaction flask was replaced by nitrogen and the vessel was tightly stoppered as soon as 1.2 cc. of chromous chloride reagent had been added. (This reagent was prepared according to the general directions of Conant and Cutter (74) as follows: 2 gm. of mossy zinc were amalgamated for 5 minutes with a solution of 150 mg. of mercuric chloride and 0.12 cc. of concentrated hydrochloric acid in 5 cc. of water, washed once with water, and added to a mixture of 5 mm of chromic chloride, 3 cc. of water, 2 cc. of concentrated hydrochloric acid, and 3 cc. of petroleum ether, and kept at room temperature overnight. A pipette fitted at the tip with a small detachable cotton filter and containing a small amount of petroleum ether was used to deliver the clear blue reagent without appreciable oxidation by air.) The reaction mixture was kept at 25° for 2 hours, diluted with ether, and washed repeatedly with water, sodium carbonate, and water. The ether phase yielded 8.0 mg. of a colorless oil which showed an absorption maximum at 240.5 m μ (ϵ = 16,400, calculated for molecular weight 416.5). Chromatographic separation over alumina with a mixture of benzene and petroleum ether (1:3 and 1:1) and with benzene gave 7.1 mg. of crystalline material which was recrystallized from dilute acetone and by concentrating a solution in a 1:10 mixture of acetone and petroleum ether (b.p. 60–70°) to incipient crystallization. 4.4 mg. of rectangular plates were obtained which melted at 160–162°.

Analysis— $C_{25}H_{36}O_5$. Calculated, C 72.08, H 8.71; found, C 71.91, H 8.70

The absorption spectrum is given in Fig. 1 ($\lambda_{\max.}$ = 240.5 m μ ; $\epsilon_{\max.}$ = 17,000).

Δ^5 -Pregnenediol-16 α ,20 α Diacetate (XI)—A solution of 17.2 mg. of Δ^5 -pregnenetriol-3 β ,16 α ,20 α 16,20-diacetate (IX) (m.p. 180.5–184.5°) in 4.8 cc. of dry, alcohol-free chloroform was kept near 0° while 45.3 mg. of phosphorus pentachloride were added in small portions (20 minutes). After 10 more minutes in the ice bath the mixture was allowed to warm up to room temperature (28°). After 1 hour the excess reagent was hydrolyzed by the addition of cold sodium bicarbonate solution. The mixture was diluted with ether and washed with water. The dry residue of the ether layer (19 mg.), which failed to crystallize, was dissolved in 4 cc. of propanol. The solution was heated under a reflux while 240 mg. of sodium were added in small portions (40 minutes). The precipitation of sodium chloride started after about 2 minutes. The crystalline reaction product (13.1 mg.) which was isolated by ether extraction was reacylated at room temperature with 2 cc. of pyridine and 1 cc. of acetic anhydride for 19 hours. The resulting acetate (17 mg.) was dissolved in petroleum ether and chromatographed on alumina. The crystalline fractions which were eluted with petroleum ether and with a 4:1 mixture of petroleum ether and benzene were preceded by 0.3 mg. of sticky semicrystalline material and followed by 4.0 mg. of an oil. The main product was recrystallized from methanol and gave 8.3 mg. of rod-shaped crystals melting at 157–159°. Occasionally the diacetate crystallized in rectangular plates.

Analysis— $C_{25}H_{38}O_4$. Calculated, C 74.59, H 9.52; found, C 74.59, H 9.49

Rotation— $[\alpha]_D^{29} = -116^\circ$ ($c = 0.26$)

3 β ,16 α -Ditritoxy-20 β -acetoxyallopregnane (III)—100.3 mg. of allopregnanetriol-3 β ,16 α ,20 β 20-monoacetate (II), 304 mg. of triphenylmethyl chloride (Eastman, purified by recrystallization from dry, thiophene-free benzene containing 20 per cent of acetyl chloride (75), and then with charcoaling from petroleum ether), and 2 cc. of dry pyridine were sealed as quickly as possible in a glass tube, heated on a steam bath for 8 hours, and kept at room temperature overnight. The tube was opened and a small piece of ice was added to the chilled reaction mixture which after 10 minutes was distributed between cold ether and ice water. The ether layer was washed twice with dilute hydrochloric acid and twice with water. These operations were carried out rapidly in a cold room with a separatory funnel containing sufficient ice to maintain the temperature near 0°. After the addition of a sodium carbonate solution the mixture was allowed to come to room temperature and shaken occasionally. After 2 hours the aqueous phase was removed, and the ether was washed with water until neutral. Some absolute ethanol was added to the ether and the solvents removed in a current of nitrogen. The dry residue (406.7 mg.) was dissolved in 2 cc. of benzene and 2 cc. of petroleum ether and passed through a column

(15.5 × 68 mm.) of Brockmann's alumina (not acid-washed). Elution with 146 cc. of a 1:3 mixture of benzene and petroleum ether gave 193.5 mg. of an oil that became opaque on the addition of ethanol. Continued washing with 40 cc. of the same solvent mixture gave 12.9 mg. of a mixture of the ditrityl ether and of tritanol, while further elution furnished tritanol which melted after three recrystallizations at 163.5–164°. The ditrityl ether which is sparingly soluble in ethanol was recrystallized from this solvent. Colorless microscopic needles were obtained which melted at 170–172°. (The melt adhered to the walls of the capillary tube and coalesced only after heating to about 190°.)

Analysis— $C_{61}H_{66}O_4$. Calculated, C 84.88, H 7.71; found, C 84.75, H 7.70

The yield was 77 per cent. If the addition of ice at the end of the reaction was omitted and the product dissolved in an alcohol-free ether instead of ordinary ether, the starting compound was recovered substantially unchanged. The reaction time and temperature were the same as were used by Riegel *et al.* (55). As the yield seemed adequate, no attempt was made to test whether these conditions are optimal in this instance.

3β,16α-Ditritoxyallopregnanol-20β (IV)—314.2 mg. of *3β,16α*-ditritoxy-20β-acetoxyallopregnane (III), 80 cc. of 95 per cent ethanol, and 15 cc. of 5 per cent aqueous sodium hydroxide were heated under a reflux for 4 hours. The mixture was concentrated and then distributed between ether and water. The ether layer was washed with water until neutral and yielded 294.1 mg. of residue. This material was used for the next step without purification. Another preparation of compound IV was recrystallized from ethanol for analysis. This product melted (again without immediate coalescence) at 168–171° and showed no depression of the melting point on admixture of the starting material (III).

Analysis—Sample dried at 100° *in vacuo*

$C_{69}H_{64}O_3$. Calculated, C 86.30, H 7.86; found, C 86.20, H 8.04

3β,16α-Ditritoxyallopregnanone-20 (V)—A mixture of 294.1 mg. of *3β,16α*-ditritoxyallopregnanol-20β (IV) in 21 cc. of acetone and of 84 mg. of chromium trioxide in 8.4 cc. of acetone was kept at 25° for 6 hours. After chilling it was distributed between 200 cc. of cold ether and 160 cc. of cold 2 per cent sodium hydroxide. The ether layer was washed once more with alkali and repeatedly with water. The ether residue (303 mg.) was freed of a small amount of amorphous impurity which was sparingly soluble in benzene or acetone. The oxidation product was dissolved in hot benzene and precipitated by the addition of ethanol. Five more recrystallizations by this procedure yielded 97.8 mg. of well formed needles melting at 271–273°. The mother liquors were chromatographed on alumina.

The first eluate (from a 3:1 mixture of petroleum ether and benzene) gave on recrystallization 5.8 mg. of ketone V; the last eluate (27.7 mg. from methanol) furnished spectrographic evidence for the presence of a hydroxyl, a keto, and a tritoxyl group. This product apparently represents partly detritylated material; it showed great tendency to separate as a gel and has not been investigated further. (Similar products were obtained in subsequent fractionations with a 4:1 mixture of benzene and ether as eluant but showed no greater tendency to crystallize.) The remainder of the material which chiefly consisted of a mixture of starting material and free tritanol was reoxidized in 21.2 cc. of acetone with 60 mg. of chromium trioxide at 25° for 6 hours and worked up as before. The total yield of ketone V was 165.5 mg. (56 per cent).

<i>Analysis</i> — $C_{30}H_{52}O_3$.	Calculated.	C 86.51,	H 7.63,	tritoxyl 63.3
	Found.	" 86.40,	" 7.55,	" 62.6

(The tritoxyl analysis was made spectrographically as described below. No correction was made for the contribution of the 20-keto group, which is not precisely known but undoubtedly small in a ditrityl compound.) The spectrum in the longer infra-red is quite similar to that shown in Fig. 2, Curve B.

Reduction of 3 β ,16 α -Ditritoxyallopregnanone-20 (V)—162.8 mg. of compound V and 6 cc. of dry ether were mixed with 96 mg. of lithium aluminum hydride in 3 cc. of dry ether. After 15 minutes of stirring under anhydrous conditions the excess of the reagent was decomposed by the gradual addition of ether which had not been freed of water or alcohol. The reaction mixture was chilled and washed with cold hydrochloric acid, with cold water, and with sodium carbonate. If the conditions described for the isolation of compound III are met, the resulting product is essentially free of tritanol. The ether residue (159.5 mg.) was dissolved in 10 cc. of acetone, mixed with 1 cc. of 1:1 hydrochloric acid, and heated under a reflux for 30 minutes. Crystals precipitated almost immediately on heating. These were separated from the cold solution and washed with acetone and ether. The more soluble material was extracted from the supernatants with ether, washed with water, taken to dryness after the addition of absolute ethanol, and combined with the precipitate (total 168.2 mg.). The reaction product was dissolved with warming in 4 cc. of pyridine and treated with 2 cc. of acetic anhydride at room temperature for 13 hours. Water was added in small portions to the chilled solution. The acetylated material was extracted with ether, washed with water and repeatedly with hydrochloric acid, sodium carbonate, and water, and after the addition of absolute ethanol taken to dryness with warming in a current of nitrogen. The dry residue (191.3 mg.) was an oil which was dissolved in 2 cc. of benzene

and 8 cc. of petroleum ether and chromatographed on a column (10.5 × 69 mm.) of alumina, as is summarized in Table V.

Triphenylmethyl Ethyl Ether (Tritoxyethane)—This compound was obtained from Fraction 1. It crystallized from petroleum ether in heavy blocks that melted at 82.5–83.5°. Smith and Smith (76) reported a melting point of 82.5–83° for tritoxylethane.

Analysis—Sample dried at room temperature *in vacuo*

$C_{21}H_{20}O$.	Calculated.	C 87.46, H 6.99
	Found.	" 87.72, " 7.13
		" 87.83, " 7.06

Absorption measurements in 95 per cent ethanol (Fig. 1) showed maxima at 259.5 $m\mu$ ($\epsilon = 717$) and at 253.5 $m\mu$ ($\epsilon = 665$) and two shoulders at 265

TABLE V

Chromatographic Separation of Acetylated Hydrolyzed Reduction Products of 3 β ,16 α -Ditritoxyallopregnanone-20 (V)

Fraction No.	Eluant		Eluate	
	Volume	Composition	Weight	Compound isolated
	cc.		mg.	
1	12	Benzene + petroleum ether 1:4	64.6	Tritoxylethane
2	85	" + " " 1:4	10.1	
3	15	" + " " 1:1	18.4	
4-6	70	" + " " 1:1	44.9	3 β ,16 α ,20 β -Triacetoxy-allopregnane (I)
7-8	75	" + " " 1:1	19.2	
9-11	78	" + " " 2:1	17.1	
12-13	37	"	8.3	3 β ,16 α ,20 α -Triacet-oxyallopregnane(VI)
14	120	"	2.2	
15	60	Methanol	4.2	

$m\mu$ and at 269 $m\mu$. The ultraviolet absorption spectrum of this compound in absolute ethanol has been reported by Anderson (77) and evidently (77) also by Orndorff *et al.* (78). The latter measurements agree substantially with our findings, while Anderson observed smaller extinction coefficients and two well resolved peaks at 266 and at 271 $m\mu$. In the longer infrared region we observed absorption maxima at 12.96, 13.16, 13.45, and 14.18 μ and an inflection near 14.35 μ . Since these bands occur at approximately the same positions also in the steroidal trityl ethers investigated, they may prove to be a characteristic of trityl ethers in general.

Triphenylmethanol (Tritanol)—Fractions 2 and 3 were recrystallized from methanol and from carbon tetrachloride and gave 21.6 mg. of tritanol melting at 161–163° and at 163–164.5° in admixture with an authentic sample (m.p. 163.5–164.5°). The spectrum of tritanol in the longer infra-

red is given in Fig. 2. Measurements below $10\ \mu$ have been reported by Barnes *et al.* (79).

Allopregnanetriol-3 β ,16 α ,20 β Triacetate (I)—Fractions 4 to 6 crystallized from methanol in platelets that seemed typical of compound I. They were therefore combined, but failed to give a pure product on recrystallization. Rechromatographing concentrated the impurity in the earlier eluates. The later fractions yielded platelets melting at 169 – 171° . These were identified as allopregnanetriol-3 β ,16 α ,20 β triacetate (I) by mixed melting point (170 – 171°) with the triacetate from mare urine and by spectrographic examination, which furnished a curve in good agreement with Curve D of Fig. 3.

Analysis— $C_{27}H_{42}O_6$. Calculated, C 70.10, H 9.15; found, C 70.27, H 9.25

Allopregnanetriol-3 β ,16 α ,20 α Triacetate (VI)—Fractions 12 to 14 crystallized spontaneously on evaporation of the eluant and gave compound VI on recrystallization. Fractions 8 to 11 crystallized in needles only upon the addition of methanol. Spectrographic examination, particularly in the regions of 10.69 and of $10.36\ \mu$, disclosed that Fractions 7 to 11 were mixtures of the two triacetates which contained decreasing proportions of the 20 β isomer. They were dissolved in small volumes of benzene and petroleum ether (1:4), reabsorbed on alumina *in the sequence of their elution*, and then eluted as before. Recrystallization of the essentially pure α fractions from methanol yielded 18.1 mg. of needle-shaped crystals which melted at 177.5 – 179.5° and at 177 – 179° in admixture with the triacetate VI (m.p. 177 – 179°) obtained by the reduction of compound VII. The infra-red spectra of both preparations are compared in Fig. 3. The addition of compound I lowered the melting point to 143 – 162° .

Analysis— $C_{27}H_{42}O_6$. Calculated, C 70.10, H 9.15; found, C 70.20, H 9.17

Rotation— $[\alpha]_D^{24} = -57^\circ$ ($c = 0.77$)

A sample of the synthetic triacetate VI was hydrolyzed and purified as described above. This sample of allopregnanetriol-3 β ,16 α ,20 α melted without discoloration at 250 – 253° . The melting point remained unchanged on admixture of Dihydrocompound A. Occasionally higher melting points (253 – 255°) were observed. A solution of the triol in 80 per cent ethanol precipitated with digitonin after 3 minutes.

3 β -Tritoxyandrostanone-17—41.9 mg. of isoandrosterone, 108 mg. of trityl chloride, and 1.3 cc. of dry pyridine were heated in a sealed tube on a steam bath for 8 hours. The mixture was worked up as described for compound III. Recrystallization from ethanol gave 67 mg. of elongated hexagonal plates which melted at 188 – 193° . Continued recrystallization raised the melting point to 193.5 – 196° .

Analysis— $C_{38}H_{44}O_2$. Calculated, C 85.67, H 8.33; found, C 85.56, H 8.33

Hydrolysis of 3 β -Tritoxyandrostanone-17—14.9 mg. of isoandrosterone trityl ether, 2 cc. of acetone, 0.1 cc. of concentrated hydrochloric acid, and 0.1 cc. of water were heated under a reflux for 30 minutes. The mixture was diluted with ether and washed with water. The ether residue (15.3 mg.) was dissolved in 0.8 cc. of benzene and 1.6 cc. of petroleum ether and chromatographed on a column (45 $\times$ 3.75 mm.) of alumina. Washing with successive portions of a 1:4 mixture of these solvents (total 28.6 cc.) gave 7.7 mg. of eluates which on recrystallization from dilute methanol furnished tritanol melting at 161–163°. Continued elution with a 1:1 mixture of benzene and petroleum ether (24 cc.) and with 10 cc. of benzene yielded 7.9 mg. of residue. This product after two recrystallizations from dilute ethanol melted at 174.5–176.5° and at 175–176° in admixture with authentic isoandrosterone. When an alcoholic solution of 3 β -tritoxyandrostanone-17 was shaken with hydrogen and the palladium-calcium carbonate catalyst of Busch and Stoeve (73) for 4 hours, a substantial portion of the starting material was recovered unchanged. The apparently more suitable palladium catalyst of Verkade *et al.* (80) was not tried.

Tritoxyl Determination—The customary assay procedures which are based on the cleavage of trityl ethers and the gravimetric determination of tritanol do not lend themselves readily to those compounds that yield alkyl moieties which resemble tritanol in their solubility behavior. Since the absorption spectra of several trityl ethers were found to be in good agreement, the spectrographic method seemed to offer a very simple substitute. Although the peak in the far ultraviolet (Fig. 1) would permit the use of very small samples, precise extinction measurements in this region are more difficult than at 260 m μ . At this wave-length the extinction coefficient ϵ/N (N = the number of trityl groups per molecule) for compound III is 731 and for 3 β -tritoxyandrostanone-17 is 750. Since the 17-keto group makes a small contribution in this region (about 12 (81)), the latter value was corrected to 738. Hence, $\epsilon/N = \frac{1}{2} (731 + 738) = 735$ was used for steroid assays (see compound V). Since a low molecular weight ether (tritoxyethane) gave a similar value ($\epsilon_{259.5} = 717$), the method may prove to be useful also in other fields in which trityl ethers with essentially non-absorbing alkyl groups are investigated. Samples of less than 1 mg. are sufficient and can be recovered unchanged. The solvent used was 95 per cent ethanol. Since one substance (V) was not sufficiently soluble to permit measurements with a 1 cm. cell in the optimal density range, a less polar transparent solvent (such as a dialkyl ether) may be preferable for the purpose.

Rotations of Known Compounds

Δ^5 -Pregnenediol-3 β ,20 α Diacetate—This compound was isolated from urine (2). M.p. 146–147°; $[\alpha]_D^{27} = -45^\circ$ ($c = 1.08$).

Allopregnenediol-3 α ,20 α Diacetate—This compound was prepared from

urinary allopregnanediol-3 α ,20 α (preparation obtained from Parke, Davis and Company through the kind cooperation of Dr. O. Kamm) by acetylation at room temperature and recrystallization from methanol. M.p. 141–142°; $[\alpha]_D^{27} = +16^\circ$ ($c = 0.91$).

Allopregnanediol-3 β ,20 α Diacetate—This compound was prepared from allopregnanediol-3 α ,20 α by epimerization (82) and acetylation at room temperature and purified by recrystallization from methanol. M.p. 168.5–169.5°; $[\alpha]_D^{25} = +2^\circ$ ($c = 0.49$).

SUMMARY

A substance (Compound A) isolated from the urine of a boy with an adrenocortical tumor has been identified as Δ^5 -pregnenetriol-3 β ,16 α ,20 α . This structure was indicated chiefly from rotation data and has been proved by the synthesis of its reduction product, allopregnanetriol-3 β ,16 α ,20 α , from the 20-epimeric triol of the urine of pregnant mares and by the oxidation of the 16,20-diacetate of the isolated compound to an α - β -unsaturated ketone. An attempt has been made to explain the results of the partial methanolysis of Δ^5 -pregnenetriol-3 β ,16 α ,20 α triacetate as a function of the configuration of the 20-hydroxyl group. It is suggested that 16-oxygenated steroids of the C₂₁ series may be derived from substances formed in the adrenal cortex.

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ANTAGONISMS IN THE UTILIZATION OF D-AMINO ACIDS BY LACTIC ACID BACTERIA

I. INFLUENCE OF DL-ETHIONINE ON THE UTILIZATION OF D-METHIONINE*

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It has been shown by Brickson *et al.* (2) and by Lyman and Kuiken (3) that leucine and valine in moderate concentrations may interfere with the utilization of D-isoleucine by *Lactobacillus arabinosus*. That aspartic acid may produce an analogous and much more marked effect on the utilization of D-glutamic acid by *L. arabinosus* has been reported by Camien and Dunn (4). Since further evidence obtained by the present authors (unpublished) has indicated that the microbiological activity of D-amino acids generally may be suppressed by specific amino acid inhibitors, it seemed desirable to initiate a series of studies on such antagonisms. The present report is concerned with the influence of DL-ethionine on the utilization of D-methionine.

EXPERIMENTAL

Medium—A dry mixture containing 1 gm. each of DL-alanine, L-arginine monohydrochloride, L-aspartic acid, L-glutamic acid, glycine, L-histidine hydrochloride monohydrate, DL-isoleucine, L-leucine, L-lysine monohydrochloride, DL-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine, and DL-valine was pulverized by hand in a glass mortar. 960 mg. of the amino acid mixture, 6 mg. of L-cysteine hydrochloride, and 5.16 gm. of Mixture B (given in Table I of Paper 65 (1)) were dissolved in distilled water and diluted to 100 ml. to prepare medium for 100 assay tubes.¹ 1 ml. of this solution was added to 1 ml. of test sample in each 13 × 100 mm. test-tube. The tubes were covered, sterilized in flowing steam at 100° for 30 minutes, and cooled. 1 ml. of sterile 6 per cent glucose solution was then added aseptically to each tube, except that a sterile

* Paper 66. For Paper 65, see Camien and Dunn (1). This work was aided by grants from the American Cancer Society through the Committee on Growth of the National Research Council. The authors are indebted to Samuel Eiduson and Gene Molene for technical assistance.

¹ The resulting final concentrations (after all solutions were added to the test-tubes) were 2 mg. per cent of L-cysteine hydrochloride and 20 mg. per cent of each of the other amino acids.

6 per cent L-arabinose solution was substituted for the glucose solution in the tests with *Lactobacillus buchneri*.² The medium was supplemented with a liver concentrate preparation³ for the tests with *Leuconostoc citrovorum*, since previous studies (6, 7) have indicated that such a supplement is necessary for good growth of this organism.

The cultures of *L. arabinosus* 17-5 (8014),⁴ *L. buchneri*, *Lactobacillus fermenti* 36 (9338), *Lactobacillus lycopersici* (4005), *Lactobacillus manni-topoeus*, *L. citrovorum* (8081), *Leuconostoc dextranicum* (8359), *Leuconostoc mesenteroides* P-60 (8042), and *Streptococcus faecalis* R (8043) were the same as those described previously (6). The inocula were prepared as described previously (8), except that L-arabinose was added to the inoculum medium for *L. buchneri*.^{2, 5} The response of each microorganism to graded amounts of L- and D-methionine was determined titrimetrically⁶ after 3 days incubation at 35°. In additional experiments with *L. arabinosus*, the response to L- and D-methionine was determined under the same conditions except for additions of DL-ethionine⁷ to the basal medium. The experimental results are given in Tables I and II.

DISCUSSION

It appeared likely that the test medium employed in the present experiments provided a balance of nutrients favorable to the utilization of D-methionine. In general, the concentrations of amino acids were much lower in this medium than in that employed in previous investigations with these organisms (6, 7). Cysteine was employed in nearly minimal

² Glucose is apparently a poor energy source for this microorganism (5).

³ A suspension of 20 gm. of liver powder (Wilson's liver concentrate powder 1:20) in 60 ml. of 10 per cent saturated aqueous ammonia was clarified in the centrifuge. 50 ml. of the supernatant were shaken with 200 ml. of water-saturated *n*-butanol acidified with 2 ml. of 96.4 per cent sulfuric acid and the mixture was centrifuged. The butanol layer was decanted immediately into 100 ml. of 10 per cent saturated aqueous ammonia. The mixture was shaken and separated in a separatory funnel. The aqueous ammonia fraction was evaporated under reduced pressure to 18 ml. and diluted with 95 per cent ethanol to 30 ml. The resulting solution, containing about 1 gm. of solids, was stored in the refrigerator. 4.8 ml. (2.5 times that needed for maximum growth of *L. citrovorum*) was added to medium for 100 assay tubes.

⁴ The numbers in parentheses are the American Type Culture Collection catalogue numbers.

⁵ 3 ml. of a sterile 6 per cent L-arabinose solution were added aseptically to 15 ml. of the sterile inoculum medium before seeding.

⁶ Growth was terminated by steaming for 20 minutes at 100°. The contents of triplicate tubes (total of 9 ml.) were combined in 50 ml. conical flasks and titrated with 0.09 N NaOH, with 3 drops of 0.8 per cent brom thymol blue in 50 per cent ethanol as the indicator.

⁷ Kindly furnished by United States Industrial Chemicals, Inc., through the courtesy of Dr. H. J. Prebluda.

concentration, and the glucose of the medium was added aseptically after sterilization. It seemed probable that certain possible methionine inhibitors, particularly those which might result from the reaction of glucose with cysteine⁸ (1), methionine (9), and other amino acids, would be lacking or at low concentrations in a medium of this composition. It may also be noteworthy that the medium contains relatively high concentrations of vitamins, including pyridoxal and pyridoxamine. The latter two vitamins have been shown to be important in the utilization of D-methionine (10) and other D-amino acids (3) by *L. arabinosus*.

All of the nine bacteria tested responded significantly to D-methionine. The response to D-methionine equaled that to L-methionine for *L. arabi-*

TABLE I

Response of S. faecalis, L. mesenteroides, and L. citrovorum to L- and D-Methionine

The values given in the table represent the ml. of 0.01 N NaOH required to titrate 1 ml. of final culture. Each value is the average of triplicate determinations.

Response to L-methionine				Response to D-methionine			
Dosage level	Organism*			Dosage level	Organism*		Organism*
	<i>S. faecalis</i> R	<i>L. mesenteroides</i> P-60	<i>L. citrovorum</i> 8081		<i>S. faecalis</i> R	<i>L. mesenteroides</i> P-60	<i>L. citrovorum</i> 8081
γ per ml.				γ per ml.			
0	1.48	1.47	4.53	0	1.48	1.47	4.53
0.67	3.44	3.58	6.88	6.7	2.62	3.31	4.94
1.33	4.68	5.71	8.80	13.3	3.27	4.32	5.10
2.00	5.42	7.49	10.30	20.0	3.87	5.36	5.30
2.67	5.88	8.88	11.55	26.7	4.38	6.05	5.50
3.33	6.19	9.70	12.56	33.3	4.79	6.88	5.82

* The six other organisms tested responded equally to L- and D-methionine. To conserve space the data for these organisms were not included in the table.

nosus, *L. buchneri*, *L. fermenti*, *L. lycopersici*, *L. mannitolpoeus*, and *L. dextranicum*. D-Methionine appeared to have only about 5 per cent of the activity of L-methionine for *L. citrovorum*, *L. mesenteroides*, and *S. faecalis* (Table I). It does not seem likely that the response of the latter organisms resulted from L-methionine present as a contaminant in the sample of D-methionine⁹ or from its partial racemization during some part of the experimental procedure, although the present data do not exclude the latter possibility. It is of interest in this connection that Steele *et al.*

⁸ It is assumed that cysteine and cystine would react similarly with glucose.

⁹ C.p. product of the H. M. Chemical Company, Ltd., Los Angeles. Specific rotation, $[\alpha]_D^{25} = -23.5^\circ$ in N HCl.

(11) have reported that D-methionine was one-tenth as active as L-methionine for *L. mesenteroides*, but had no activity for *L. citrovorum*. *S. faecalis* showed substantial utilization of D-methionine in media buffered with citrate, but not in media buffered with acetate, according to the report of Lyman and Kuiken (3).

The effects of DL-ethionine on the utilization of L- and D-methionine by *L. arabinosus* are illustrated in Table II. DL-Ethionine at a concentration of 21 mg. per cent (the lowest concentration tested) effected a

TABLE II

Effect of DL-Ethionine on Utilization of L- and D-Methionine by L. arabinosus

The values given in the table represent the ml. of 0.01 N NaOH required to titrate 1 ml. of final culture. Each value is the average of triplicate determinations.

Methionine	Methionine enantiomorph tested					
	L	D	L	D	L	D
	DL-Ethionine concentration					
	None		21 mg. %		42 mg. %	
<i>γ per ml.</i>						
0	15.80		2.99		3.03	
1	17.69	17.72	7.50	4.09	8.16	3.68
2	18.30	18.18	11.29	7.91	11.20	4.48
3	18.67	18.60	13.22	11.99	13.11	5.51
5	18.70	18.60	15.93	15.02	15.90	10.38
Methionine	DL-Ethionine concentration					
	167 mg. %		333 mg. %		667 mg. %	
	<i>γ per ml.</i>					
0	2.63		1.58		1.16	
1	8.32	2.53	5.81	1.48	1.39	1.18
2	11.02	3.58	8.39	1.60	4.42	1.25
3	12.89	3.81	10.37	1.53	5.83	1.12
5	15.58	5.03	10.75	2.00	8.88	1.19

marked reduction in the blank titration¹⁰ and slightly reduced the relative activity of the D-methionine. Further additions of DL-ethionine up to 167 mg. per cent resulted in progressively greater reductions in D-meth-

¹⁰ The high methionine blanks obtained with *L. arabinosus* under the stipulated conditions were not unexpected, since it has been shown that this organism possesses only a partial requirement for methionine (6), although under other conditions reasonably low methionine blanks may be obtained with this organism (10). The blank titrations with the organisms for which data are not given in Tables I and II ranged from 0.87 to 1.80 ml.

ionine activity with only slight changes in response to L-methionine. Higher concentrations of DL-ethionine tested (maximum, 667 mg. per cent) further decreased the relative activity of D-methionine and also markedly reduced the response to L-methionine. These relationships appear to be entirely analogous to those found previously with L- or D-aspartic acid and D-glutamic acid for *L. arabinosus* (4).

Since it appears that D-methionine may be converted to L-methionine before it is utilized by *L. arabinosus* (10), it seems likely that a methionine inhibitor, such as DL-ethionine, might interfere with this conversion as well as with the utilization of the resulting L-methionine. The results given in Table II were not entirely unexpected, therefore, because of the additional inhibition which was predicted for the D-methionine. It is evident from the data, however, that the relationship between DL-ethionine and either L- or D-methionine was not truly competitive.

It was concluded from the present investigations that, under adequate conditions, D-methionine (and by analogy perhaps certain other D-amino acids) may be as active as the corresponding L form for a number of lactic acid bacteria. The inhibitions of D-methionine by ethionine (present data), D-glutamic acid by aspartic acid (4), D-isoleucine by leucine and valine (2, 3), and D-alanine by glycine (authors' unpublished data) indicate that the greater reduction by specific amino acid inhibitors of D-amino acid activity than of L-amino acid activity for lactic acid bacteria probably is a general phenomenon. This generalization may be limited to cases in which the D-amino acid is not an essential metabolite. Details of investigations on comparable antagonisms involving D-alanine, D-aspartic acid, D-phenylalanine, and other D-amino acids will be given in the papers to follow in this series.

SUMMARY

D-Methionine was utilized as well as was L-methionine by *Lactobacillus arabinosus*, *Lactobacillus buchneri*, *Lactobacillus fermenti*, *Lactobacillus lyopersici*, *Lactobacillus mannitopoeus*, and *Lactobacillus dextranicum* under the stipulated experimental conditions. D-Methionine was partially utilized by *Leuconostoc citrovorum*, *Leuconostoc mesenteroides*, and *Streptococcus faecalis*. The relative activity of D-methionine compared with L-methionine for *L. arabinosus* decreased progressively with increasing concentrations of DL-ethionine in the test medium. The response of *L. arabinosus* to L-methionine was affected little by changes in DL-ethionine concentration from 21 to 167 mg. per cent, whereas the response to D-methionine was decreased with each increase in DL-ethionine concentration. Higher concentrations of DL-ethionine (333 and 667 mg. per cent) markedly depressed the response to L-methionine as well as to D-methionine. The

hypothesis was proposed that the greater reduction by specific amino acid inhibitors of D-amino acid activity than of L-amino acid activity for lactic acid bacteria is a general phenomenon.

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THE MECHANISM OF THE DIABETOGENIC ACTION OF URIC ACID

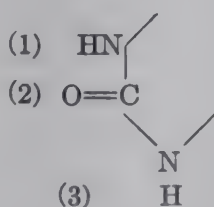
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Injection of alloxan lowers the reduced glutathione (GSH) of tissues (1) and of blood (2, 3). Recently Lazarow (4) has shown that simultaneous injection of glutathione prevents the diabetogenic action of alloxan. Substitution of alloxan in one imino group at position 1 by a benzyl or by a butyl radical or in two imino groups at positions 1 and 3 by methyl radicals abolishes the diabetogenic properties of the molecule (3). However, benzyl- and butylalloxans both lower blood GSH, as does ninhydrin (3) which is also non-diabetogenic (3, 5). It would appear that the triketo grouping which is common to alloxan and the homologues mentioned, and to ninhydrin, is responsible for the decrease in GSH. According to various authors (3, 6, 7), alteration of the triketo grouping of alloxan by substitution at the C₅ position also abolishes diabetogenic activity, but Gitter and Cardeza (8) report that diabetes follows injection of barbituric or violuric acid.

The inference of the above evidence is that when glutathione in the islands of Langerhans is lowered by the triketo grouping of alloxan the configuration



is necessary for the appearance of diabetes. This suggests that a purine with a configuration at positions 1, 2, and 3 similar to alloxan, such as uric acid, might be diabetogenic if injected into animals previously depleted of sulfhydryl. Leaf and Neuberger (9) have shown that liver GSH can be lowered by feeding a methionine- and cystine-deficient diet. This work implied that the increase in potency of alloxan in animals on a methionine- and cystine-deficient diet (10) is due to a general depletion of sulfhydryl including blood GSH. Accordingly, it was found by feeding rabbits the methionine- and cystine-deficient diet described by Haag and Wright (11) that blood GSH could be lowered to a level comparable to that brought

about by injection of alloxan (about 25 mg. per cent). With this level as a criterion of general sulfhydryl deficiency, it was found that injection of uric acid induced a hyperglycemia and glycosuria lasting 4 to 5 days (12). Addition of 0.2 per cent methionine to the diet maintained the blood GSH above 30 mg. per cent. Injections of uric acid then had no diabetogenic effect.

The above observations suggest that necrosis of the β -cells in the islands of Langerhans may occur in uric acid diabetes as it occurs in alloxan diabetes. Lazarow (4) has suggested that the necrosis brought about by alloxan may be due to inhibition of sulfhydryl enzyme systems in the β -cells.

This paper is a report of some experiments to determine whether or not the islands of Langerhans are involved in uric acid diabetes and to determine the effect of uric acid on the activity of the sulfhydryl enzyme hexokinase. The diabetogenic potencies of other oxypurines and pyrimidines are also compared.

EXPERIMENTAL

*Animals*¹—Australian wild rabbits and inbred hooded rats were used. The rabbits were obtained as kittens from burrows and raised in the laboratory until 600 to 800 gm. in weight.

Diets—The Haag and Wright diet used in the preliminary work (11) has been further modified. The per cent composition of the modified diet is as follows: starch 76.57 to 71.57, peanut meal 10.00 to 15.00, Hubbell's salt mixture 3.50,² peanut oil 5.00, cod liver oil 4.00, wheat germ oil 0.70, vitamin K 0.01, ascorbic acid 0.10, vitamin B (exclusive of biotin and folic acid but including choline; sufficient biotin and folic acid present in peanut meal) 0.12. For convenience this is called the sulfhydryl-deficient diet.

In general, rabbits fed this diet for 4 to 5 weeks have blood GSH values of 24 mg. per cent or less. However, the blood GSH in some rabbits may take up to 7 weeks to fall to this level. Ascorbic acid was added, since Levey and Suter (13) found that the diabetogenic effect of alloxan was increased by this substance.

¹ The attempts to make sulfhydryl-deficient rats diabetic with uric acid (up to twelve injections each of 1 gm. per kilo) were not successful. Blood GSH was lowered by eliminating protein from the diet altogether. The liver of both injected and control rats showed pycnosis of nuclei, fatty infiltration, and sometimes focal necrosis. These lesions may have prevented the appearance of diabetes.

² Composition of salt mixture expressed in gm.: CaCO_3 , 543; MgSO_4 , 16.0; MgCO_3 , 25.0; NaCl , 69.0; KCl , 112.0; KH_2PO_4 , 212.0; $\text{FePO}_4 \cdot 4\text{H}_2\text{O}$, 20.5; KI , 0.08; MnSO_4 , 0.35; NaF , 1.00; $\text{Al}_2(\text{SO}_4)_3$, K_2SO_4 , 0.17; CuSO_4 , 0.90.

By increasing the amount of peanut meal to 50 per cent at the expense of starch, it was found that blood GSH was maintained above 30 mg. per cent. This is called the sulfhydryl-sufficient diet.

Injections—All purines and pyrimidines tested, with the exception of alloxan, were injected intraperitoneally as suspensions or solutions in distilled water. The concentration was about 10 per cent weight per volume. If diabetes failed to occur after one injection, a second was given 72 to 96 hours after the first. Alloxan was injected intravenously.

Criteria of Diabetes—Rabbits were considered to be diabetic only if the blood sugar rose to 200 mg. per cent or more, and if glycosuria occurred 2 to 6 days after the last injection. When diabetes was established, the animals were fed a standard laboratory diet and fresh alfalfa.

Chemical—Blood sugar was determined by the Hagedorn-Jensen method and blood GSH by the method of Woodward and Fry (14). Nylander's test was used to detect glycosuria, as uric acid gives a positive reaction with copper reduction methods.

Assay of Pancreatic Insulin—Total pancreas tissue was removed and extracted, and crude insulin prepared by the method of Jephcott (15). The crude insulin was taken up in $N/300$ HCl and the potency determined by a crossover test on four rabbits by the method of Marks and Pak (16). The pancreas of sulfhydryl-deficient rabbits was removed after they had been on the diet 5 weeks. Those of the rabbits on the sulfhydryl-sufficient diet were excised after 6 weeks. The pancreas of uric acid and alloxan diabetic rabbits was removed 4 to 5 days after the last injection.

Hexokinase Activity of Muscle Extracts—Extracts were made by mincing rat muscle with 3 volumes of ice-cold distilled water in a Waring blender and filtering through a Whatman No. 41 filter paper on a Büchner funnel. These extracts were used either fresh or after dialysis against cold distilled water for 4 to 5 hours. In the latter case guanine hydrochloride was added to the reaction mixture to a concentration of 1.0×10^{-5} M. Dihydrocozymase need not be added, as it is not removed during dialysis if the pH is kept between 7.0 and 8.0 (17).

The complete test system (volume 2.4 cc.) was as follows: 0.47 cc. of extract, 0.015 M NaHCO_3 , 0.005 M MgCl_2 , 0.004 M adenosine triphosphate, 0.0058 M glucose, and 0.06 M NaF. The reaction time was 40 minutes; temperature 38° ; pH 7.5.

The hexokinase activity of this system was determined by the decrease in P_7 (inorganic phosphate after hydrolysis for 7 minutes in 1 N HCl) by methods described elsewhere (18).

Cytological—Pancreas tissue was stained by the method recommended by Richardson (19).

Results

Diabetogenic Potencies of Oxypurines and Pyrimidines—From the data summarized in Table I it is apparent that uric acid was diabetogenic in sulfhydryl-deficient rabbits and that under the same conditions uracil and xanthine were not. Caffeine and theobromine were obviously too toxic to be of any use in the study of diabetes.

The negative result with uracil and xanthine was surprising, especially since Gitter and Cardeza (15) found that barbituric and violuric acids were diabetogenic. It would appear, then, that the diabetogenic properties of uric acid are not solely due, as originally thought, to the presence of the configuration found at positions 1, 2, and 3 in the molecule. These proper-

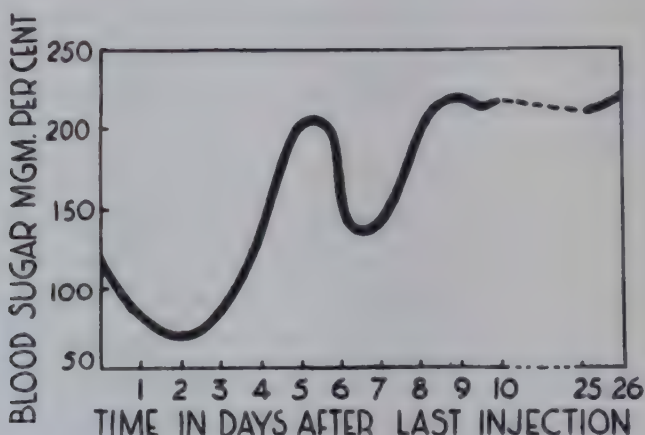


CHART 1. Effect of two intraperitoneal injections of uric acid, each of 1 gm. per kilo, on the blood sugar levels of a sulfhydryl-deficient rabbit. Nylander's test for glycosuria was positive from the 9th day onward. The standard laboratory diet and fresh alfalfa were fed from the 8th day onward.

ties of uric acid may be due to the properties of the molecule as a whole, or may be due to the formation of alloxan from uric acid.

From Table I, it is seen that two rabbits out of twelve failed to become diabetic after two injections of uric acid. These were given a third injection without result. Uric acid was not diabetogenic in rabbits fed the sulfhydryl-sufficient diet.

In general, uric acid diabetes lasted 5 to 10 days. However, of the ten rabbits with uric acid diabetes, one, which had received two injections, showed a slow onset of diabetes, but the hyperglycemia and glycosuria persisted until the animal was killed about 3 weeks later (Chart 1). Cytological examination of the islands of Langerhans showed lesions similar to those found in the islands of rabbits with mild alloxan diabetes (blood sugars less than 250 mg. per cent). These lesions were pycnosis of nuclei and marked degranulation of the β -cells (Fig. 1). The islands of a rabbit

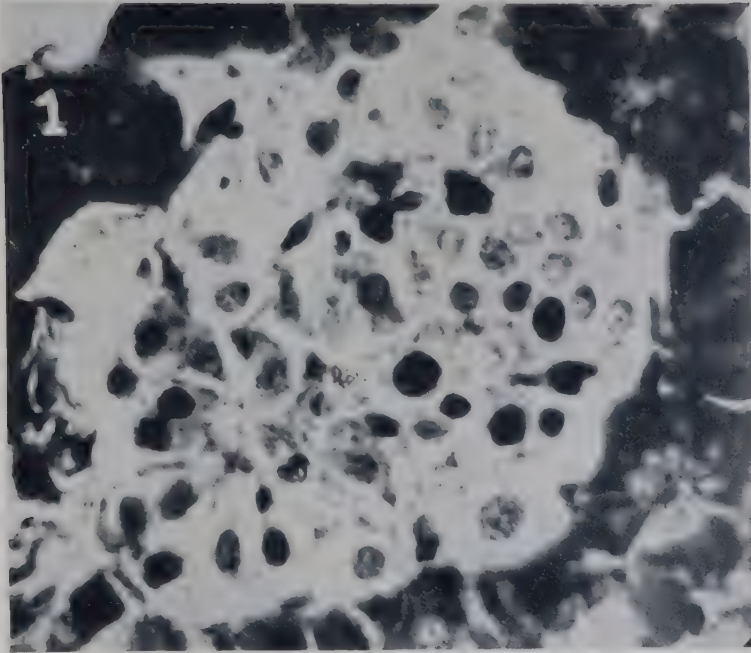


FIG. 1. Islands of Langerhans in the pancreas of a rabbit made diabetic with uric acid. The nuclei are pycnotic and the cytoplasm is degranulated in the β -cells. $\times 1000$.

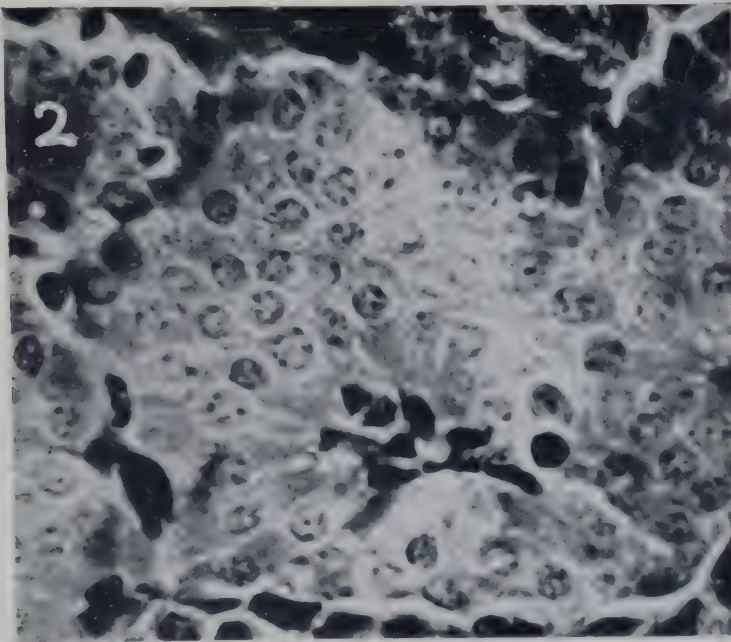


FIG. 2. Islands of Langerhans in the pancreas of a sulfhydryl deficient rabbit which had received two intraperitoneal injections of uracil, each of 1 gm. per kilo. The nuclei are normal but some of the β -cells are degranulated. $\times 1000$.

injected with uracil were relatively normal, but some β -cells showed degranulation (Fig. 2).

The insulin content of sulfhydryl-deficient rabbits was markedly decreased (Table I). This is not surprising, since a diet deficient in cystine and methionine would lead to general protein deficiencies of which lack of insulin (rich in cystine) is one manifestation. A decrease in the insulin content of rats on a diet in which gelatin was the protein has been observed by the Toronto school (20).

Apparently the rabbits on the sulfhydryl-deficient diet were becoming diabetic before injection with uric acid, as the insulin content of uric acid diabetes was little, but not significantly, less than that of uninjected rabbits. The insulin content of the rabbits on the sulfhydryl-sufficient

TABLE I

Diabetogenic Properties of Oxypurines and Pyrimidines in Rabbits on Various Diets

Diet	No. of animals	Average blood GSH	Substance injected	Dose	No. of injections	Incidence of diabetes	Insulin content of pancreas* per 100 gm. body weight
		mg. per cent		gm. per kg.			units
Sulfhydryl-deficient	2	23	Caffeine	0.44	1	Death within 2 min.	
	2	24	Theobromine	0.44	1	Death within 1-3 days	
	4	23	Xanthine	1	2	Nil	
	4	22	Uracil	1	2	"	
	12	23	Uric acid	1	1-2	10	0.36
	3	23	None			Nil	0.42
Sulfhydryl-sufficient	6	32	Uric acid	1	2	"	0.95
Standard laboratory + fresh alfalfa	3	40	None			"	1.20
	3	38	Alloxan monohydrate	0.2	1	3	0.30

* Average of three organs.

diet was substantially normal, even after injection with uric acid. The figure for the insulin content of rabbits with alloxan diabetes is in good agreement with that for uric acid diabetics, and from the general biochemical and cytological picture it would seem that Shaw Dunn diabetes and uric acid diabetes are similar. However, it is possible that the hyperglycemia following injection of uric acid is due to impaired liver function. It is well known that liver damage can occur on a methionine- and cystine-deficient diet and the fact that the blood sugar returned to normal a few days after feeding a normal diet could be interpreted as an improvement of liver function. However, the facts that the insulin content of the diabetic rabbits was markedly decreased and that one rabbit was per-

sistently diabetic on the normal diet argue that the diabetes is pancreatic in origin.

Effect of Oxypurines and Pyrimidines on Hexokinase Activity—The capacity of alloxan to inhibit sulfhydryl enzymes is well known and Lazarow (4) has suggested that alloxan produces diabetes because of inactivation of essential sulfhydryl enzymes in β -cells. He postulates that the β -cells have a low glutathione content as a result of the cysteine of the glutathione incorporated into the insulin molecule as cystine. It must be remembered, however, that alloxan has other properties such as deamination of α -amino acids and ability to increase respiration enormously (21). These as well as —SH oxidation may be involved in the process of killing β -cells.

It is obviously difficult to study enzyme inhibition in the islands of mammals and even in fish islands, which can be obtained reasonably free of acinar tissue, since α - and γ -cells occur in addition to β -cells (22). How-

TABLE II

Influence of Uric Acid, Uracil, Xanthine, and Alloxan on Transfer of Phosphate from Adenosine Triphosphate to Glucose in Fresh Extracts of Rat Muscle

HCl of pH 3.6 was used to control the low pH of uric acid, alloxan, and uracil. The results are the average of two experiments. P₇ 332.5 γ at zero minute.

Additions	P ₇ at 40 min.	P transfer
	γ	γ
HCl.....	265.0	67.5
4.4×10^{-4} M uric acid.....	260.0	72.5
4.4×10^{-4} " xanthine.....	268.5	64.0
4.4×10^{-4} " uracil.....	265.0	67.5
4.4×10^{-4} " alloxan.....	268.0	64.5
2.86×10^{-3} M ".....	305.5	27.0

ever, the effect of structurally related diabetogenic and non-diabetogenic substances on a sulfhydryl enzyme, such as muscle hexokinase, is instructive. It has been found (18) that alloxan can inhibit muscle hexokinase 100 per cent, while ninhydrin inhibits 80 per cent. Cysteine releases the alloxan inhibition completely and partially releases the inhibition by ninhydrin. If this represents what happens to sulfhydryl enzymes in β -cells, it is possible that the greater degree of inhibition by alloxan results consistently in the death of the cells. It is interesting to note here that Stoll (23) finds that alloxan and ninhydrin produce similar lesions in the pancreas of guinea pigs and rats.

The possibility that uric acid and other purines and pyrimidines might inhibit hexokinase has been tested by adding the compounds to extracts of rat muscle. The results are summarized in Table II.

From these figures it is apparent that, in a concentration of 4.4×10^{-4}

M, uric acid, alloxan, xanthine, and uracil had no inhibitory effect on the hexokinase activity of fresh muscle extracts. However, alloxan in a concentration of 2.86×10^{-3} M brought about substantial inhibition. Results similar to the above were obtained with extracts in which glutathione had been lowered by dialysis, although the activity of these extracts was much reduced.

Owing to the insolubility of uric acid and xanthine, it was not possible to increase their concentrations above the level of 4.4×10^{-4} M. However, the effects of these substances on purified enzymes will be studied; presumably, smaller concentrations of inhibitors will be far more effective. Until this is done the question of whether or not the uric acid molecule as a whole possesses biochemical properties similar to alloxan remains open.

Attempts to show the presence of a uricase in water extracts of acetone-dried liver (rabbit) capable of catalyzing the reaction, uric acid $\rightleftharpoons$ alloxan, were not successful.³ Nevertheless, the possibility that the reaction occurs cannot be discounted, for Ascoli and Izar (25), in a foot-note to their paper, state that the reaction, uric acid $\rightleftharpoons$ dialuric acid, occurs in liver and blood.

DISCUSSION

Young (26) has recently discussed the relationship of experimental diabetes to clinical diabetes and concluded that "clinical" diabetes is not a single entity. In view of this it seems reasonable to suppose that uric acid could be of importance in the etiology of some types of diabetes in an organism lacking uricase in its tissues, as in man. The work of Thorn *et al.* (27) and of Conn *et al.* (28, 29) supports this view. If such is the case, the clinical implications of the observation that enough of even a poor protein like peanut meal protects against the diabetogenic action of uric acid are obvious. Among other possibilities the protein may protect simply by providing amino acids for the synthesis of insulin or by the synthesis of glutathione. The similarity in structure of alloxan and uric acid argues that the maintenance of glutathione is the important factor, as insulin will not protect against the diabetogenic action of alloxan (6).

Further evidence is found in the observations that guinea pigs have relatively high blood GSH values and are resistant to the initial hypo-

³ Alloxan was determined by the colorimetric and fluorimetric methods described by Archibald (24). The extracts were freed of ascorbic acid and glutathione by dialysis against cold distilled water. The reaction was carried out at pH 5.2 in the presence of adenosine triphosphate and magnesium. The low pH was considered necessary, as alloxan decomposes to alloxanic acid at a higher pH. The protein was precipitated by trichloroacetic acid which was neutralized later. Recoveries of added alloxan from the extracts were good (90 per cent or more).

glycemic and diabetogenic actions of alloxan (30, 31). Further experiments have shown that guinea pigs are still resistant to the diabetogenic action of alloxan when fed the sulfhydryl-deficient diet for 2 weeks. If they are left longer on the diet, the blood GSH can be lowered to about 40 mg. per cent, but they succumb within a few hours to the hypoglycemic and toxic effects of alloxan.

It is apparent from this work and the experiments with uric acid that a compound cannot be described as without diabetogenic action without reference to the level of blood GSH. Even alloxan is without diabetogenic action in guinea pigs with high blood GSH levels.

SUMMARY

Uric acid is diabetogenic in rabbits whose blood GSH is lowered to about half the initial value by feeding a methionine- and cystine-deficient diet. Under the same conditions xanthine and uracil are not diabetogenic.

Uric acid is not diabetogenic in rabbits whose blood GSH is maintained within normal limits by feeding the methionine- and cystine-deficient diet modified by inclusion of extra protein.

Cytologically, uric acid diabetes is similar to mild alloxan diabetes. The insulin content of rabbits with uric acid diabetes and of rabbits fed a methionine- and cystine-deficient diet is reduced to about one-third of that of normal rabbits. The insulin content of rabbits fed extra protein is substantially normal.

Uric acid, xanthine, uracil, and alloxan, in a concentration of 4.4×10^{-4} M, do not inhibit hexokinase activity in fresh and dialyzed extracts of rat muscle. 2.86×10^{-3} M alloxan causes a substantial inhibition of hexokinase activity.

Addendum—After this paper was presented for publication, Collins-Williams and Bailey (32) reported that a single injection of uric acid caused transient hyperglycemia in three of sixteen rabbits whose blood GSH had been lowered by dietary means or by bleeding. They found that their rabbits did not tolerate the methionine- and cystine-deficient diet well and nine out of twenty-one rabbits died while on the régime. They observed that in eight of the surviving rabbits blood hemoglobin had fallen from an initial average level of 9.5 gm. per 100 cc. (range 5.4 to 11.3) to 5.8 gm. per 100 cc. (range 3.8 to 7.9).

It was considered of interest to determine the hemoglobin of Australian wild rabbits which were used in my own experiments. In a series of six rabbits selected at random from the colony, the average hemoglobin value was 14.2 gm. per 100 cc. (range 13.4 to 14.8). The hemoglobin was determined by the method of Gibson and Harrison (33), the standard being checked against a sample of blood, the hemoglobin of which had been determined by a spectrophotometric method.

It seems that, compared to wild rabbits, animals of the type used by Collins-Williams and Bailey are mildly anemic on a normal diet. It would appear that robust

rabbits must be used, and more than one injection of uric acid be given in order to induce uric acid hyperglycemia consistently.

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THE DETERMINATION OF HYDROXYPROLINE

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There has been a great need for a satisfactory method for the determination of hydroxyproline to facilitate the study of the composition of proteins. Lang (1) and Waldschmidt-Leitz and Akabori (2) developed a colorimetric estimation involving oxidation to pyrrole with sodium hypochlorite and color formation with isatin or *p*-dimethylaminobenzaldehyde. However, oxidation was said to be incomplete and a correction factor was necessary. Dakin (3) and Bergmann (4) estimated hydroxyproline by isolation procedures. These procedures also required the use of large correction factors, as well as relatively large quantities of protein. McFarlane and Guest (5) devised a colorimetric method for hydroxyproline involving sodium peroxide oxidation and color formation with copper and isatin. This method yielded low values according to Devine (6), and in our hands similar results were obtained.

The unique high hydroxyproline content of collagen suggests the desirability of an accurate method for the determination of this amino acid in small quantities as a means of estimating the amount of collagen or gelatin in a mixture of proteins. Because hydroxyproline so far has not been found to be a nutritive requirement for a microorganism, a chemical procedure is required.

A short and simple colorimetric method is here described that is applicable to the determination of hydroxyproline in hydrolysates of 40 to 100 γ of collagen with a reproducibility of ± 2 per cent (Table II) and an accuracy of ± 2 per cent (Table I) as judged by recovery of hydroxyproline from elastin hydrolysates and from an amino acid mixture simulating collagen. Oxidation, in the manner of McFarlane and Guest with sodium peroxide, yields products that form an intense red color with *p*-dimethylaminobenzaldehyde. The intensity of color produced with 5 to 15 γ of hydroxyproline is 4 to 5 times that formed in previous colorimetric procedures. Because of this greater intensity of color and because of a different preliminary treatment of the hydroxyproline solutions, only 1 to 2 per cent as much protein is required as in earlier methods.

In acid hydrolysates of proteins, the only amino acid other than hy-

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droxyproline which gives color under the conditions employed is tyrosine, which yields 1.5 per cent as much color as does hydroxyproline. This interference is so small that it can be corrected from the tyrosine content of the protein. Pure tryptophan gives 0.7 per cent as much color as hydroxyproline but humin formation on acid hydrolysis of proteins eliminates this interference.

Reagents—

Standard solutions of L-hydroxyproline containing 5, 10, and 15 γ of hydroxyproline per ml. Pfanstiehl c.p. hydroxyproline was found to be satisfactory.

0.01 M copper sulfate solution.

2.5 N sodium hydroxide.

6 per cent hydrogen peroxide.

3.0 N sulfuric acid.

5 per cent *p*-dimethylaminobenzaldehyde in c.p. *n*-propanol. The *p*-dimethylaminobenzaldehyde is recrystallized according to the procedure of Adams and Coleman (7), followed by recrystallization from warm alcohol by adding water and cooling.

Procedure

For the assay of one unknown solution, nine dry 18 $\times$ 150 mm. Pyrex test-tubes are employed. With an accurate pipette, the reagents are distributed as follows: Tube 1, 1 ml. of distilled water; Tubes 2 and 3, 1 ml. of standard containing 5 γ of hydroxyproline; Tubes 4 and 5, 1 ml. of standard containing 10 γ of hydroxyproline; and Tubes 6 and 7, 1 ml. of standard containing 15 γ of hydroxyproline. 1 ml. of the solution to be assayed, containing 5 to 15 γ of hydroxyproline, is pipetted into the eighth and ninth tubes.

Into each test-tube is pipetted in succession 1 ml. each of 0.01 M copper sulfate solution, 2.5 N sodium hydroxide, and 6 per cent hydrogen peroxide.

The solutions are mixed and shaken occasionally during a period of 5 minutes, and are then placed in a water bath at 80° for 5 minutes with frequent vigorous shaking. The heating and shaking destroy the excess of peroxide. Traces of peroxide which remain will decrease color formation and produce an orange-red hue. The tubes are chilled in an ice and water bath and 4 ml. of 3.0 N sulfuric acid are added with agitation; 2 ml. of *p*-dimethylaminobenzaldehyde solution are then added with thorough mixing.

The tubes are placed in a water bath at 70° for 16 minutes and then cooled in tap water. The contents are transferred to selected absorption tubes and light transmission is read with an Evelyn photoelectric colorimeter and a No. 540 filter. Tube 1 is used for the blank setting.

The amount of hydroxyproline (measured in micrograms) in 1 ml. of unknown solution is established by finding the point corresponding to its optical density on the standard curve prepared at the same time. Per cent of hydroxyproline in the sample of material is determined by the equation

$$\frac{\text{Micrograms of hydroxyproline in 1 ml. of unknown solution}}{\text{Micrograms of sample in 1 ml. of unknown solution}} \times 100$$

Protein hydrolysates are prepared by autoclaving 50 mg. of protein with 1.0 ml. of 6 N HCl in sealed tubes for 3 hours at 50 pounds pressure. The hydrolysates are neutralized, brought to appropriate volume, and filtered if necessary. Assay values were 93 per cent of maximum after 1 hour of hydrolysis. Maximum values were reached after 3 hours and did not decrease after 8 hours hydrolysis. As described in Table I, hy-

TABLE I
Recovery of Hydroxyproline

Substance added	Hydroxy- proline added	Added hydroxyproline recovered			
		1	2	3	4
	γ	γ	γ	γ	γ
0.....	13.0	12.9	13.0		
105 γ amino acid mixture*.....	13.0	13.1	13.3	13.3	13.1
53 " " " " *.....	6.5	6.6	6.5		
500 " cattle hemoglobin.....	13.0	12.5	12.6	12.7	
500 " casein.....	13.0	12.6	12.6	12.5	
540 " pig aorta elastin†.....	6.5	6.6	6.4		

* As in collagen save for hydroxyproline.

† Contained 1.50 per cent hydroxyproline.

droxyproline is quite stable when subjected to the conditions of hydrolysis for 3 hours.

Because this procedure is intended to be used for the estimation of collagen in mixtures of proteins including animal tissues, the proteins were dried for 16 hours at 108° and weighed in glass-stoppered weighing bottles with precautions to prevent the absorption of moisture. The varied hygroscopic properties of tissues or protein mixtures of unknown composition make this the method of choice. In order that the results might be referred to other preparations of the same proteins, nitrogen determinations were made with a precision of ± 0.4 per cent on more than fifty portions of assorted gelatins, collagens, and elastins weighed in this manner.

Gelatins, collagens, and elastins were prepared as previously described (8).

Results

Fig. 1 shows a standard curve prepared with 0 to 20 γ of hydroxyproline. The light absorption is 4 to 5 times that produced by the isatin reaction (5) referred to earlier.

Hydroxyproline was added to hemoglobin, casein, pig aorta elastin, and to a mixture of amino acids corresponding to the composition of collagen save for hydroxyproline. These mixtures, as well as hydroxy-

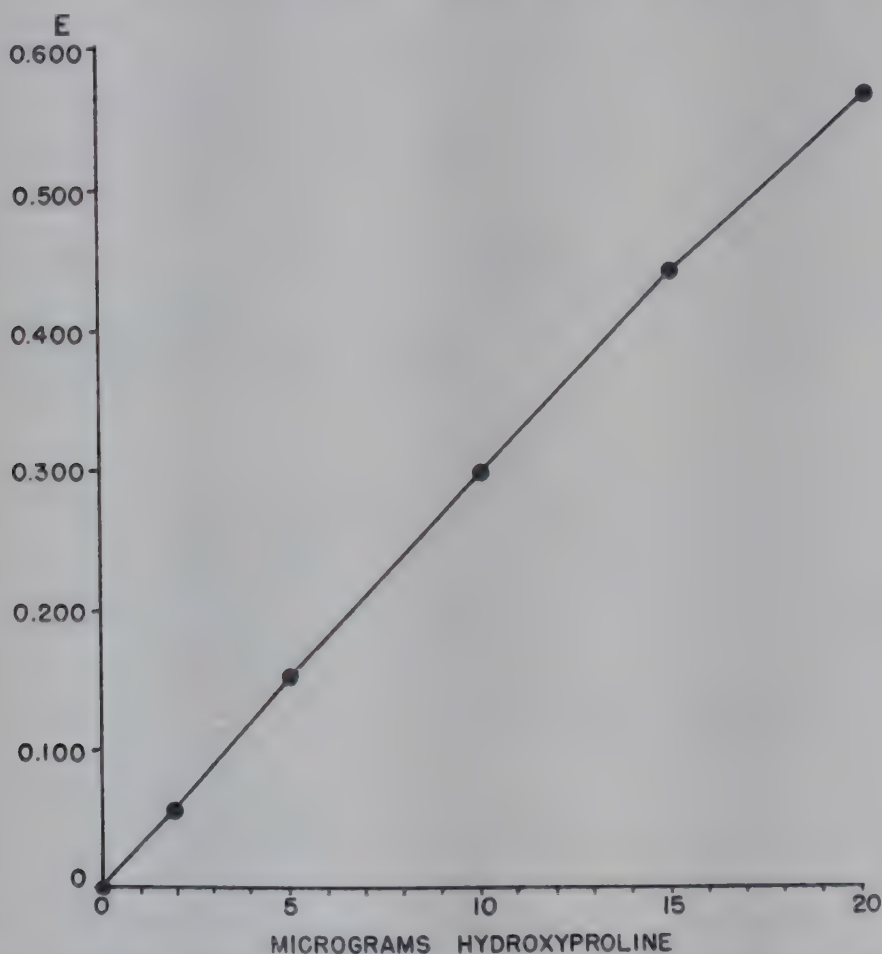


FIG. 1. The formation of color with hydroxyproline. $E = 2 - \log$ of per cent light transmission with a No. 540 filter.

proline alone, were subjected to the usual procedure for preparation of hydrolysates (in 6 N HCl, 3 hours at 50 pounds pressure) and hydroxyproline was determined (Table I). Hydroxyproline was completely recovered after autoclaving with elastin, with amino acid mixtures simulating collagen, or by itself in the hydrochloric acid. However, small losses appeared after autoclaving hydroxyproline with 37 times its weight of hemoglobin or casein. These latter hydrolyses may represent conditions

which are more severe than would be encountered in any anticipated use of the procedure because the entire quantity of hydroxyproline is free to react with substances liberated during the hydrolysis of the casein or hemoglobin. It may be presumed that hydroxyproline originally in peptide combination in these proteins would be partially protected during hydrolysis and recovery would be nearer the theoretical value. If the procedure is to be used for the estimation of collagen in tissues, a preliminary separation of the collagen is anticipated.

Guest (9) reported that proline oxidized by sodium peroxide in the presence of copper sulfate gave 10 per cent as much color with *p*-dimethylaminobenzaldehyde as did hydroxyproline. It is possible that the proline was contaminated with hydroxyproline. Three commercial preparations of L-proline were found by us to yield color corresponding to 2.3 to 3.4 per cent hydroxyproline. Upon formation of the crystalline complex with cadmium chloride as described by Kapfhammer and Eck (10), the color formation decreased to 1 per cent hydroxyproline. After recrystallizing the complex four times from hot water by adding alcohol and cooling, removing the cadmium by hydrogen sulfide, and evaporating the remaining solution so that the proline crystallized, the proline gave color corresponding to 0.17 per cent hydroxyproline.

Hydrolysates of proteins (casein, zein, cattle hair, wool) containing a high content of proline gave no indication of color formation due to proline. It is evident that the method here described gives no significant amount of color with proline.

Although three commercial samples of L-proline were contaminated with hydroxyproline, two different commercial samples of L-hydroxyproline were found by microbiological assay with *Leuconostoc citrovorum* 8081 to be free from proline.

Tyrosine appears to be the only interfering substance commonly encountered in protein hydrolysates prepared as described. Under the conditions of the determination, tyrosine yields 1.5 per cent as high readings as hydroxyproline with a No. 540 filter. The color produced with tyrosine may be differentiated from the hydroxyproline color by its bronze hue with maximum absorption at 500 $m\mu$ instead of at 550 $m\mu$, by its much greater stability, and by its greater solubility in amyl alcohol. Casein, lactalbumin, ovalbumin, cattle hemoglobin, fibrin, zein, wool, silk fibroin, cattle hair, chicken epidermal scales, and turtle scutes yielded traces of color that displayed the properties of the color formed with tyrosine by the assay procedure. The absorption with the No. 540 filter corresponded to 0.01 per cent (hemoglobin) to 0.23 per cent (turtle scutes) hydroxyproline. After correction for tyrosine content, the hydroxyproline equivalent was negligible.

Pure tryptophan yields color corresponding to 0.7 per cent hydroxyproline. However, interference of tryptophan present in proteins is eliminated by the formation of humin during acid hydrolysis. To proteins containing large amounts of tryptophan but forming little humin, glucose may be added before hydrolysis to insure destruction of the tryptophan. 20 mg.

TABLE II
Hydroxyproline Content of Gelatins, Collagens, and Elastins

Protein*		Hydroxy- proline	S. e.†	No. of de- terminations
		gm. per 100 gm. protein		
Gelatins	Bacto-difco	13.6	0.144	4
	Eastman purified calf skin	14.4	0.138	6
	" " pig "	13.1	0.076	4
	Fish scale	10.3	0.047	4
Collagens	Cattle hide‡	13.2	0.129	6
	" " §	13.3	0.087	4
	" bone	14.1	0.087	4
	" Achilles tendon	13.4	0.166	6
	Pig Achilles tendon	13.5	0.148	6
	Sheep Achilles tendon	13.7	0.145	7
	Chicken tarso-metatarsal tendon	13.5	0.158	4
	Cattle tail tendon	13.3	0.112	4
	Rat tail tendon	13.0	0.075	4
	Kangaroo tail tendon	13.0	0.029	4
	Fish skin	9.1	0.029	4
Elastins	Cattle ligamentum nuchae	1.84	0.028	4
	Sheep " "	0.84	0.014	3
	Cattle " " **	1.92	0.026	4
	" aorta**	1.91	0.004	4
	Pig aorta**	1.50	0.015	4
	Sheep aorta**	1.46	0.029	4

* These proteins were prepared as previously described (8).

† Standard error = $\sqrt{(\Sigma(X - \bar{X})^2)/(N(N - 1))}$.

‡ Preparation involved treatment with trypsin and $\text{Ca}(\text{OH})_2$.

§ Preparation involved treatment with 10 per cent NaCl and acetone.

|| Preparation involved heating 24 hours in water at 100° .

** Preparation involved heating 40 hours in 40 per cent urea at 100° .

of glucose were sufficient to eliminate entirely the color produced from 25 mg. of tryptophan added to 50 mg. of casein before hydrolysis. This treatment has no effect on hydroxyproline or tyrosine.

Table II shows the hydroxyproline content of gelatins, collagens, and elastins as determined by the present method. It will be noted that the hydroxyproline content of most gelatins and collagens was 13 to 14 per

cent (13 to 14 gm. of hydroxyproline per 100 gm. of dry weight of protein). These values are within the limits of the "best" values in the literature. The range of these values in the literature is 12.9 to 14.6 per cent of gelatin (1, 3-5, 11). It will be noted that fish skin collagen and fish scale gelatin yielded outstandingly low values. In a previous publication (8) it was reported that fish skin collagen was higher in threonine, serine, and methionine than collagen of higher organisms. It is significant that the hydroxyproline content of fish skin collagen is proportionally lower than in the other collagens. Fish scale gelatin was also found to be high in threonine and methionine and somewhat high in serine (8); the hydroxyproline content again was low.

The elastin from ligamentum nuchae and aorta of cattle contained about 1.9 per cent hydroxyproline, in accordance with the value reported by Stein and Miller (12). However, pig and sheep aorta elastin contained 1.5 per cent hydroxyproline and sheep ligamentum nuchae elastin, 0.8 per cent. Previous assay of other amino acids of pig elastin has revealed several differences in its composition from other elastins (8). At this time it cannot be said whether these differences are due to impurities.

We are indebted to Dr. R. M. Lollar and Dr. P. R. Buechler of the Tanner's Council Laboratory, University of Cincinnati, for the two samples of hide collagen.

SUMMARY

A procedure for the colorimetric determination of hydroxyproline is described.

The hydroxyproline content of four gelatins, eleven collagens, six elastins, and several other preparations is reported.

Gelatin and collagen from mammalian and avian sources contained 13.0 to 14.4 per cent hydroxyproline.

Gelatin and collagen from fish contained 9.2 to 10.3 per cent hydroxyproline.

Elastin from ligamentum nuchae and aorta of cattle contained 1.84 to 1.92 per cent hydroxyproline. Elastins from pig and sheep tissues were lower, possibly owing to impurity.

Several other proteins examined appeared to contain no hydroxyproline.

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INGESTION OF HISTIDINE AND THE GLUTAMIC ACID BLOOD LEVEL IN RATS*

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The metabolism of histidine has been extensively studied, but our knowledge of the catabolism of histidine is still far from complete. Histidine is known to be essential for growth, but the course of metabolism in excess of that required for growth is uncertain. Most of the information available on histidine has dealt with growth, excretion of metabolites, or nitrogen balance. A few studies have been made of the changes in the amino, urea, and non-protein nitrogen content of blood after an amino acid was fed (1-4). Buck and Berg (5) have followed the changes in tryptophan and urea and non-protein nitrogen content of the blood after tryptophan was fed. Crookshank and Berg (6) followed the changes in the histidine, total imidazole, urea, amino, and non-protein nitrogen of the blood of rats following the oral administration of both D- and L-histidine. They concluded that L-histidine probably was not converted to other imidazole derivatives, but that the imidazole ring was ruptured with the formation of a metabolite retaining the α -amino nitrogen. This is in keeping with the theory that L-histidine is converted to glutamic acid during the process of metabolism (7). Featherstone and Berg (8), while observing the metabolism of histidine by kidney and liver slices, noted that L-histidine might be converted to other metabolites than glutamic acid. They also concluded that histidase played a more important rôle than L-amino acid oxidase in the metabolism of histidine. With D-histidine they noted that the D isomer was not attacked by histidase and is not readily oxidized by D-amino acid oxidase. In their blood studies, Crookshank and Berg (6) could not find any evidence of conversion of the D isomer to other imidazole derivatives or the rupture of the imidazole ring.

It seemed to us that examination of the blood both before and at intervals after feeding single doses of D- or L-histidine for changes in the glutamic acid, histidine, total imidazole, and amino nitrogen content might produce further evidence of the pathway of histidine metabolism.

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EXPERIMENTAL

The L-histidine used in these studies was obtained by recrystallization of a commercial preparation. The D-histidine was prepared from DL-histidine essentially as outlined by Conrad and Berg (9). 2 per cent solutions of the monohydrochloride monohydrate gave $[\alpha]_D^{20} = +8.30^\circ$ for L and -8.26° for the D isomer. Analyses for amino and for total nitrogen gave essentially theoretical values.

Experimental animals were stock rats which were maintained on laboratory chow. Their weights ranged from 150 to 275 gm., usually between 200 and 240 gm. The animals were fasted for 24 hours before the experimental period was begun and then fed, by stomach tube, 100 mg. of histidine, as the monohydrochloride monohydrate, per 100 gm. of body weight. This is a little more than can be absorbed in 1 hour (10).

Blood was drawn by heart puncture 1 to 12 hours after L-histidine was fed and 1 to 6 hours after the D isomer was fed. The animal had previously been given intraperitoneally 5 mg. of pentobarbital, sodium (nembutal) per 100 gm. of body weight to produce anesthesia in 3 to 5 minutes. The blood was transferred immediately from the syringe to an oxalated container. To provide the minimum amount of blood for all determinations, it was necessary to withdraw 8 to 10 ml. of blood. As a result, an animal could be used for only one specimen and not over a period of several hours.

The blood was divided into two portions for the precipitation of the proteins. One portion was treated with 7.9 volumes of 0.12 N sulfuric acid and 1.1 volumes of 10 per cent sodium tungstate to provide the protein-free filtrate used in the determination of glutamic acid. The precipitated proteins were removed by centrifugation. The protein-free filtrate was adjusted to a pH of 6.8 by the addition of 2 to 4 drops of dilute sodium hydroxide. The small amount of sodium hydroxide required does not appreciably affect the volume. 4 volumes of 5 per cent trichloroacetic acid were added to the other portion to give the filtrate used in the estimation of histidine, total imidazole, and amino nitrogen. Crookshank and Berg (6) have shown that the trichloroacetic acid filtrate is preferable to the tungstic acid filtrate for determinations involving histidine because the tungstic acid apparently precipitates some of the histidine.

Histidine was determined by a modification of the relatively specific (10) Kapeller-Adler method (11). Total imidazoles were estimated by a modification of the Theis-Benedict phenol method (12), which was used in conjunction with the Kapeller-Adler method to determine whether histidine was being converted into other imidazoles. Amino nitrogen was determined manometrically according to the procedure of Van Slyke (13). Glutamic acid was determined microbiologically with *Lactobacillus arabinosus*.

sus. The assay medium was modified from that of Hier *et al.* (14) by substituting DL-aspartic acid for asparagine and omitting DL-norleucine, L-hydroxyproline, and glycine. When L-glutamic acid was added to this medium, it would support growth equally as well as the original medium of Hier *et al.* (14). The solution for assay was prepared by adding to 1.0 ml. of assay medium 1.0 to 2.0 ml. of tungstic acid filtrate and sufficient distilled water to make a final volume of 3.0 ml., autoclaving for 10 minutes at 15 pounds pressure and allowing to cool to room temperature. 1 drop of an innoculum of *L. arabinosus*, prepared in the usual manner, was then added and the mixture was incubated for 72 hours at 37° and titrated with standard 0.05 N sodium hydroxide with bromothymol blue as the indicator. The assay solutions were compared with standard solutions containing known amounts of glutamic acid. Blanks were run on all solutions. All assay, standard, and blank solutions were prepared in quintuplicate. The assay organisms were kept as stab cultures in an agar, tryptone, beef extract, glucose, and water medium, and were transferred every 7 days to a fresh stab, incubated for 24 hours, and stored in the refrigerator. The innoculum medium was the same as that used for the stabs, except that agar was omitted.

RESULTS AND DISCUSSION

The averaged results for L-histidine are shown in Fig. 1. The data are the average of six to ten blood specimens for the first 6 hours, and of four thereafter.

No histidine could be detected in the blood of fasted animals, but it appeared promptly after L-histidine was fed, reached a maximum in 1 hour, dropped rapidly, and completely disappeared in 6 hours. The total imidazoles, calculated as histidine, closely paralleled the changes in histidine. When corrected for the imidazoles found in the fasting animal, the imidazole values become almost identical with those for histidine. If L-histidine were converted to other imidazole derivatives, the process would appear to occur too slowly to be detected by our method. This is in keeping with the results previously reported (6).

The amino nitrogen, after reaching peak concentration in 1 hour, returned essentially to its initial fasting level at the end of 6 hours. At its peak, histidine could account for only about 50 per cent of the increase found in amino nitrogen at that time. The difference seems sufficient to warrant the assumption either that histidine is converted into a metabolite which retains amino nitrogen or that, under the stimulus of histidine, amino nitrogen is produced from some other source. This is in agreement with previous results (6) and is consistent with the theory that glutamic acid is produced during histidine metabolism (7).

HISTIDINE CONVERSION IN BLOOD

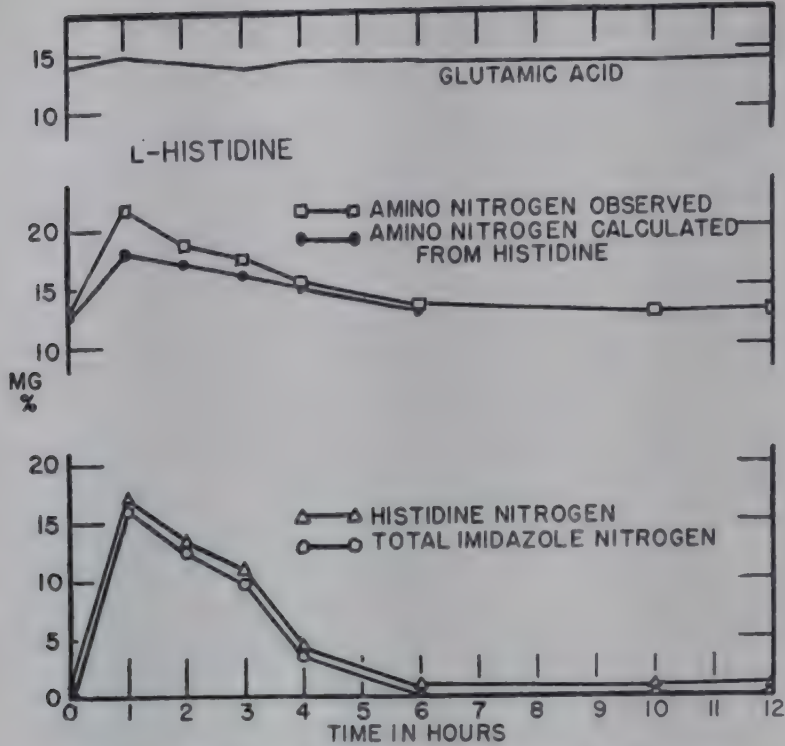


FIG. 1. Effect of L-histidine on blood components

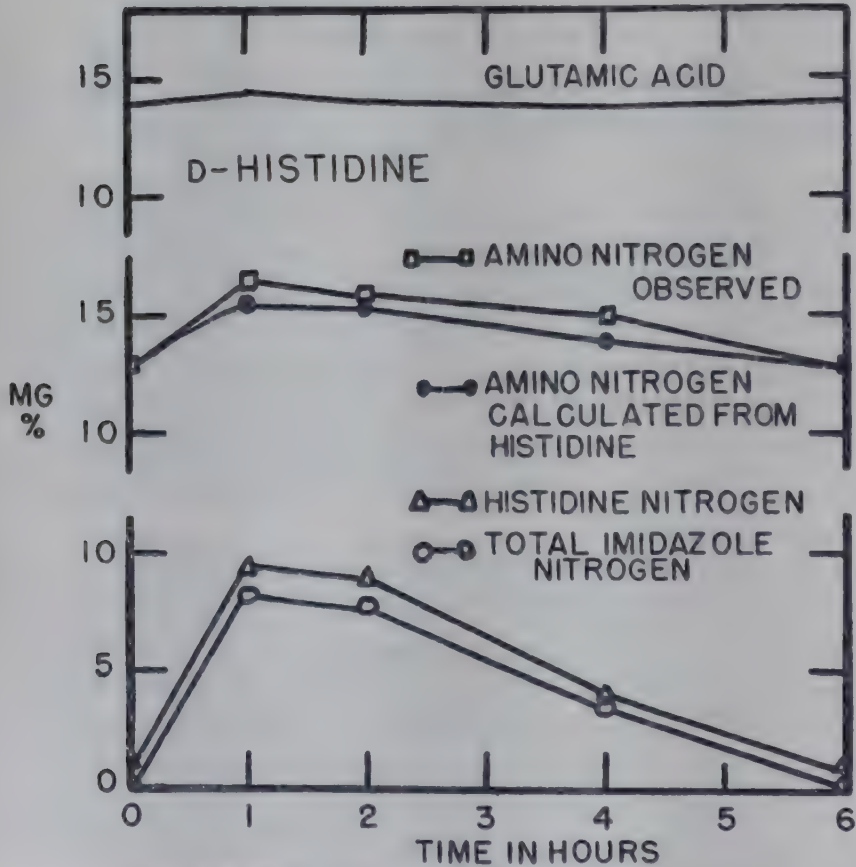


FIG. 2. Effect of D-histidine on blood components

The glutamic acid level in the blood did not show any significant increase over the 12 hour period. This would indicate either that there was no conversion of L-histidine to glutamic acid or that the process occurred too slowly to be detected by our method.

The averaged results for D-histidine are shown in Fig. 2. The results are the average of four to six blood specimens.

In no instance was the increase in blood levels after ingestion of D-histidine as great as with the L isomer, even though the rate of absorption of the two from the gut is approximately the same (10). The histidine and total imidazole levels closely paralleled each other. If D-histidine is converted to other imidazole derivatives, the process probably occurs too slowly to affect the imidazole concentration of the blood.

The amino nitrogen showed an immediate increase, reached its peak in 1 hour, and returned to its base level in 6 hours. The difference between the amino nitrogen found by assay and the increase calculated from histidine was much less marked than with L-histidine. The difference is so small that it probably is of doubtful significance. This would suggest that the D isomer is not readily acted upon. These results are in agreement with those previously reported (8).

Glutamic acid did not show any appreciable increase during the experimental period. Thus it would appear that there was no conversion of histidine to glutamic acid or that it was produced too slowly to be detected.

SUMMARY

After L-histidine was fed to rats previously fasted for 24 hours, the changes in the histidine, total imidazole, amino nitrogen, and glutamic acid content of the blood indicated that either no conversion to glutamic acid occurred, the conversion proceeded too slowly to be detected by our method, or any glutamic acid formed was rapidly converted to some other metabolite retaining the α -amino nitrogen. In analogous studies with D-histidine, the changes indicated that there was no conversion of D-histidine to other imidazole derivatives and that no rupture of the imidazole ring occurred.

L-Histidine produced a much greater increase in the histidine, total imidazole, and amino nitrogen content of the blood than did the D isomer.

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CRYSTALLINE ALCOHOL DEHYDROGENASE FROM BAKERS' YEAST*

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Alcohol dehydrogenase, the enzyme which catalyzes the reversible oxidation of alcohol to acetaldehyde, was purified and crystallized from brewers' yeast by Negelein and Wulff (1). The procedure described by these authors is laborious and time-consuming, and, particularly with American brewers' yeast, the yield is small.

It is the purpose of this paper to report the isolation and crystallization of alcohol dehydrogenase from bakers' yeast by a simple procedure resulting in a yield of about 50 per cent of the enzyme activity present in the maceration juice. The crystalline enzyme can be obtained from the maceration juice within 1 working day. The procedure has been repeated in two other laboratories with similar success.¹ The enzyme is a useful tool for the study of dismutation reactions and for the determination of diphosphopyridine nucleotide (DPN), and has also been used as a model for kinetic studies of DPN-linked enzyme reactions.

EXPERIMENTAL

Preparation of Crystalline Alcohol Dehydrogenase—Fleischmann's bakers' yeast was obtained in 1 pound cakes, crumbled, and dried between two sheets of paper in a thin layer for 4 to 5 days at room temperature. The dry yeast was finely ground in a ball mill at 0° for 16 hours and stored in the cold room in a well stoppered container. 200 gm. of the dry yeast powder were extracted with 600 ml. of 0.066 M disodium phosphate for 2 hours at 37° with continuous stirring, followed by extraction at room temperature for an additional 3 hours. The yeast residue was then removed by centrifugation at 13,000 r.p.m. for 15 minutes.² The supernatant solution was quickly brought to 55° and maintained at this temperature in a water bath for 15 minutes; after cooling, the mixture was centrifuged. At this stage the clear supernatant fluid can be stored at 0° overnight.

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¹ Personal communication from Dr. A. Kornberg, National Institute of Health, and Dr. S. Korkes, New York University College of Medicine.

² The residue can be reextracted at least twice with considerable yields of alcohol dehydrogenase, which can be carried through to the crystalline state by the same procedure used for the first extract.

To each 100 ml. of the yeast extract, 50 ml. of ice-cold acetone were slowly added, while the temperature of the mixture was maintained at about -2° in a dry ice-alcohol bath. The resulting precipitate was separated by centrifugation at 0° and discarded. To the supernatant solution, 55 ml. of cold acetone were added for each 100 ml. of the yeast extract, the temperature being kept at -2° . The mixture was centrifuged at 0° and the supernatant solution discarded. The precipitate was suspended in about 50 ml. of cold water and dialyzed for 3 hours against running tap water. The precipitate was removed by centrifugation. To the clear supernatant solution 36 gm. of solid ammonium sulfate were added per 100 ml. of solution. After standing at 0° for 30 minutes, the mixture was centrifuged at 15,000 r.p.m. for 20 minutes at 0° . The precipitate was dissolved in 20 ml. of distilled water and 4 gm. of ammonium sulfate were added. The precipitate was centrifuged off and discarded. From

TABLE I
Purification of Alcohol Dehydrogenase from Bakers' Yeast

	Total units	Specific activity	Yield
		units per mg. protein	per cent
1st extract*.....	55,000,000†	3,700	
After heating.....	62,000,000		100
Acetone ppt.....	56,000,000	55,000	90
1st crystalline preparation.....	38,000,000	120,000	61
Recrystallized preparation.....	26,000,000	158,000	42

* Prepared from 200 gm. of dried yeast.

† The activity measurements of the first extract are usually low, probably due to side reactions occurring during the test.

the supernatant solution, the alcohol dehydrogenase enzyme started to crystallize a few minutes after the further addition of ammonium sulfate, which was added in small portions during the course of several hours until about 60 per cent of ammonium sulfate saturation was reached. The crystals were collected by centrifugation and resuspended in a small volume of distilled water, from which they started to recrystallize almost immediately on addition of small volumes of saturated ammonium sulfate solution. The recrystallized material was dialyzed against distilled water for 2 hours with rapid stirring and was then lyophilized. A typical protocol showing data on the specific activity of the various fractions and the yields obtained is presented in Table I.

The dried enzyme has been stored for several weeks in a vacuum desiccator with little loss in activity. According to experiments described by

A. Kornberg,³ the enzyme can be stored in the frozen state and can be thawed repeatedly without marked loss of activity. For most of the studies reported in this paper, a solution of the lyophilized preparation was used.

Spectrophotometric Measurement of Enzymatic Activity—The enzymatic activity of alcohol dehydrogenase was determined in a Beckman model DU quartz spectrophotometer with limiting amounts of enzyme and an excess of substrate. The enzyme preparation, diluted in 0.01 M phosphate buffer, pH 7.5, containing 0.1 per cent solution of gelatin or serum albumin to avoid surface denaturation, was added to a solution containing 0.01 M ethyl alcohol and 0.00005 M diphosphopyridine nucleotide. The final volume was 3 ml. and the final pH 8.8 with 0.01 M sodium pyrophosphate as buffer. The control cell contained all the solutions except the substrate. The changes in optical density at 340 m μ were recorded at intervals of 15 seconds. 1 unit is defined as a change in $\log I_0/I$ of 0.001 per minute. The increment in density, $\log I_0/I$, between the reading at 15 and 45 seconds after mixing of the solution, multiplied by 2, is taken as the enzyme activity per minute. Although the rate of DPN reduction falls off with time, the initial rates are proportional to the enzyme concentrations used.

Determination of Diphosphopyridine Nucleotide (DPN)—In the presence of an excess of alcohol dehydrogenase (100 γ) and a large excess of alcohol (1 mM), the reduction of DPN is very rapidly completed. The optical density obtained is directly proportional to the amount of DPN added and can be used to determine the DPN concentration in unknown solutions. From the equilibrium data presented later in this paper, it becomes apparent that, within the limits of experimental error, at an alkaline pH and in the presence of excess alcohol, DPN becomes fully reduced. This is confirmed by the fact that addition of semicarbazide, as used by previous investigators (1, 2) for the completion of the reaction, does not increase the amount of reduced DPN. Since DPN enters into this reaction stoichiometrically, at least 5 γ of DPN must be present to obtain an accurate reading. DPN, even in amounts below 1 γ , can be measured by means of alcohol dehydrogenase and yellow enzyme or diaphorase with semicarbazide as aldehyde fixative (1, 2). Enzymatic methods of DPN determination have the advantage of simplicity and specificity, in contrast to those methods depending on phosphorus determination or chemical reduction by hydrosulfite.

Equilibrium Studies with Alcohol and Lactic Dehydrogenase—The use of alcohol dehydrogenase for the quantitative determination of diphosphopyridine nucleotide, in the absence of aldehyde fixatives such as semicarbazide, required a consideration of the equilibrium of the reaction.

³ Personal communication.

Previous studies (3-5) have revealed a striking effect of pH on the equilibrium reaction catalyzed by enzymes requiring DPN. Negelein and Wulff (1) reported an equilibrium value, $([\text{alcohol}] [\text{DPN}_{\text{ox.}}])/([\text{aldehyde}] [\text{DPN}_{\text{red.}}]) = 1350$ at pH 7.9. No measurements at other pH values are recorded in their paper.

The reaction catalyzed by alcohol dehydrogenase can be written as follows:



It is apparent from this formula that hydrogen ions take part in the reaction and, therefore, that the pH at which the reaction is carried out will markedly affect the final equilibrium. High hydrogen ion concentration should favor the equilibrium of the reaction to the left, while low hydrogen ion concentration should shift the equilibrium to the right.

To determine quantitatively the effect of H^+ concentration on the equilibrium of the reaction, activity measurements were made at different pH levels. Measurements were carried out under the following conditions: 0.1 mg. of alcohol dehydrogenase, $30 \mu\text{M}$ of sodium pyrophosphate, $0.151 \mu\text{M}$ of diphosphopyridine nucleotide, and $16.2 \mu\text{M}$ of ethyl alcohol were added, and the pH adjusted by the addition of dilute NaOH or HCl. The final volume was 3 ml. The pH was measured with a glass electrode at the end of each experiment. The equilibrium constants were determined by the following equations:

$$k = \frac{[\text{acetaldehyde}] [\text{DPN}_{\text{red.}}]}{[\text{alcohol}] [\text{DPN}_{\text{ox.}}]} \quad (1)$$

$$k_{\text{H}} = \frac{[\text{acetaldehyde}] [\text{DPNH}] [\text{H}^+]}{[\text{alcohol}] [\text{DPN}^+]} \quad (2)$$

From the absorption at $340 \text{ m}\mu$, the values for reduced DPN were calculated from the molecular absorption coefficient (6). For each molecule of DPN reduced, 1 molecule of alcohol is oxidized to acetaldehyde. The aldehyde concentration, therefore, is equal to the concentration of reduced DPN. The value for oxidized DPN was obtained by subtracting the amount reduced from the amount added. The alcohol concentration remained virtually constant, since less than 1 per cent could be oxidized by the amount of DPN present.

When the k values were plotted on semilogarithmic paper against the pH at which the reaction took place, a straight line with a slope of 1 was obtained (Fig. 1). This can be readily understood from equation (2) as written above, in which the H^+ ion concentration is included in the equilibrium formula. A shift of 1 pH unit (which is equivalent to a 10-fold

change in H^+ concentration), as expected, changes the value for the equilibrium constant by 10-fold. If the equilibrium is calculated with the inclusion of the H ion concentration, a constant k_H is obtained at different pH values. For alcohol dehydrogenase, $k_H = 1.15 \times 10^{-11}$. The same relationship was found for lactic acid dehydrogenase⁴ at different pH values (see Fig. 1). The k_H value $= 4.4 \times 10^{-12}$.

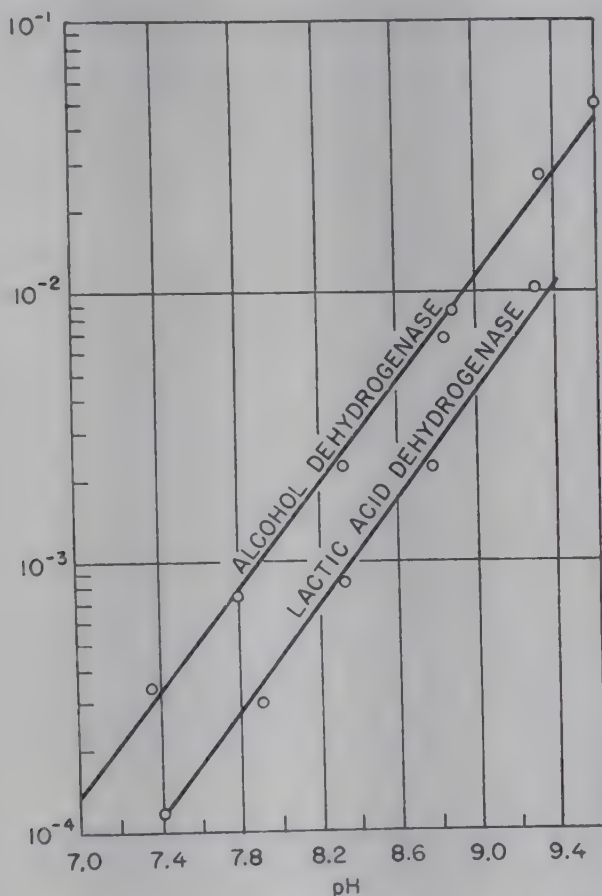


FIG. 1. Effect of pH on the equilibrium constant k for alcohol and lactic acid dehydrogenase. The values for k are plotted on the ordinate.

DISCUSSION

Alcohol dehydrogenase with semicarbazide as an aldehyde fixative and in conjunction with yellow enzyme was first used for DPN determinations by Negelein and Wulff (1). From the equilibrium studies presented in the present paper it is apparent, however, that, at alkaline pH and with an excess of alcohol, DPN becomes fully reduced within the limits of experimental error without the use of substances which shift the equilib-

⁴ A lactic acid dehydrogenase preparation of 40 per cent purity, prepared from rabbit muscle, was used for these studies.

rium. Thus at pH 8.8, 0.1 μM of DPN is more than 98 per cent reduced in the presence of 1 μM of ethyl alcohol and an excess of alcohol dehydrogenase.

The equilibrium data presented above show that coenzyme-linked reactions, such as those catalyzing the oxidation of alcohols to aldehydes or of lactate to pyruvate, can be measured directly in the spectrophotometer from either side of the reaction. Such measurements can also be made if the equilibrium appears unfavorable, provided the enzyme is not easily denatured in the pH range at which the test must be carried out. Both alcohol dehydrogenase and lactic acid dehydrogenase retained sufficient activity, even at a pH of 9.3. Since a great excess of enzyme can be used for these experiments, considerable losses in enzyme activity do not alter the final results of the equilibrium studies.

Perhaps a few words of caution should be added in regard to the equilibria data. The use of an enzyme to obtain equilibria data presumes that the enzyme acts as a perfect catalyst and that the equilibrium reached is independent of the nature of the enzyme used. This was shown to be true for the succinate-enzyme-fumarate equilibrium measured by Borsook and Schott (7), but was not demonstrated in the above study. However, the amounts of alcohol dehydrogenase used are very small as compared to the substrate and the product of the reaction, and therefore are not likely to affect the equilibrium materially. The above reported results are in good agreement with the data previously obtained by Negelein and Wulff (1) with crystalline alcohol dehydrogenase and by Kubowitz and Ott (8) with crystalline lactic acid dehydrogenase.⁵ However, all these data show considerable variation from the results obtained with crude enzyme preparations (3, 4).

For these reasons, it should be emphasized that the values obtained and the equation used may hold only for the particular conditions used in these experiments.

SUMMARY

1. A simple method for the isolation of crystalline alcohol dehydrogenase from Fleischmann's bakers' yeast is described.
2. The enzyme is suitable for enzymatic determination of diphosphopyridine nucleotide.
3. Data on the equilibrium of the reaction catalyzed by the enzyme at different pH values are presented.

⁵ In that paper it was shown that with higher temperatures the equilibrium values for the lactate-pyruvate system increase. The values reported in this paper, which were determined at 25°, are slightly higher than those of Kubowitz and Ott at 22°.

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TOLUIDINE BLUE BINDING BY DEVELOPING MUSCLE TISSUE: ASSAY AND DATA ON THE MECHANISM INVOLVED*

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The correlation of the basophilia of tissues with their nucleic acid content is one of the oldest and most widely applied chemical interpretations of histological staining reactions. The interaction of basic dyes with tissue constituents has been regarded for a long time, and without explicit qualification, as a salt formation. This interpretation was based essentially on the observation of stoichiometric relations between the maximum combining capacities of proteins with dye ions, as in the experiments of Schmidt (1). The limitations of such an oversimplified explanation soon became evident; a clear distinction (2) between various types of forces which participate in these reactions seems to provide a much wider possibility of experimentation and make more concrete the interpretation of results. With increasing knowledge of the reaction mechanism of dye combination, this type of reaction can be used as a quantitative assay as well as a sensitive indicator for changes in the properties of the reacting groups. A study of the interaction of dye cations and nucleoproteins was attempted by Hammarsten *et al.* (3). More recently, Kelley and Miller (4) and Michaelis (5) studied the metachromatic changes in the spectral properties of some basic dyes on combination with nucleic acids. Pischinger (6) and his followers have shown how biological materials depend upon the pH and the dissociation of the substrate. This development has been reviewed and subjected to detailed criticism in a noteworthy report by Levine (7). Later this use of dyes was expanded in numerous contributions by Dempsey, Wislocki, and Singer (8), and others. An interesting series of experiments on the interaction of basic dyes with nucleoproteins after treatment with various fixing agents was carried out by Kelley (9) in continuation of his studies with pure nucleic acids. Brachet (10) used the uptake of basic dye to assay ribose nucleic acid in the cytoplasm of developing cells, controlling his observations by comparing tissue slices before and after treatment with ribonuclease. He also attempted a quantitative determination of dye after extraction from the tissues and a correlation of dye uptake with analytical data for nucleic acid content

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(11). The action of ribonuclease in connection with staining techniques has recently been reviewed by Stowell and Zorzoli (12).

The present investigation is a quantitative study of the combining capacity of the basic dye, toluidine blue, with elements of developing muscle tissue. (a) Nucleic acid content of developing rat muscle, between the 14th day of gestation and 10 days post partum (13), is correlated with dye-binding capacity of this tissue at different pH values. A strict relationship between nucleic acid values and amounts of dye fixed was found to exist over only a part of the pH range examined. (b) Tissue from which nucleic acid was removed was similarly studied. (c) Combination of the dye with pure nucleic acid in solution and with mixtures of nucleic acids and proteins was also measured.

EXPERIMENTAL

For the determination of toluidine blue uptake by muscle tissue, samples of 200 to 500 mg. were dissected from rats at different stages of development, following a procedure described previously (14). The earliest developmental stage used was that of 12 days gestation, followed by 5 day intervals to 10 days after birth. The dissected tissue was minced with scissors, weighed on a micro torsion balance, and homogenized in a glass homogenizer for 3 minutes with 1 ml. of ice-cold distilled water for each 100 mg. of wet weight. 0.1 ml. portions of the homogenate were spread over two-thirds of the surface of a 1 × 3 inch microscope slide and dried at about 70° for 1 hour. The slides were cooled and placed in 400 ml. Pyrex beakers filled with a solution of toluidine blue (National Aniline and Chemical Company, Inc.; certified), which contained 158 mg. of the commercial preparation (59 per cent dye content) in 500 ml. of distilled water. The solution of the dye was facilitated by heating in a water bath. It was prepared at least 24 hours before use, and its pH was adjusted to 6.8, 4.2, 3.8, and 3.2. The slides with the dried homogenate were immersed in the dye solution for 3 hours. Tests showed that no more dye is taken up after this period. Not more than six slides were stained at any one time in each beaker in order that an excess of the dye solution should be available for each slide. After staining, the excess dye was drained and each slide was rinsed with distilled water until traces only of stain were observable in the wash fluid. The back of the slide and its edges were carefully wiped with filter paper and the slides were allowed to dry at room temperature. The dye was extracted with 10 ml. of a solution made up of 2.5 ml. of concentrated H_2SO_4 and 2.5 ml. of water in 95 ml. of methanol. After extraction, the tissue shows only a faint coloration. The quantity of dye remaining in the tissue is negligible compared to the amounts extracted. The dye content of the extracts was compared with standard solutions of toluidine blue which were made up in the same

solvent. The four calibration standards contained 58, 29, 14.5, and 7.3 γ of toluidine blue in 10 ml. The samples of the extract were matched by dilution with the standard samples. Although the semilogarithmic plot of the colorimetric values for the standards was close to a straight line, the above precautions eliminated small deviations from Beer's law. These deviations are known to occur in solution of many dyes because of polymerization. The readings were carried out at first with an Evelyn colorimeter at 660 $\mu\mu$, later on in a Cenco-Sheard spectrophotometer. Since the maximum absorption of toluidine blue is at 635 $\mu\mu$, determination at 660 $\mu\mu$ reduces the absorption to about two-thirds maximum.

In the course of the determinations, several sources of technical errors appeared. One of these is the use of the common Coplin staining jars. The inferior quality of the glass in these jars causes a rapid drift of the pH of unbuffered and even of weakly buffered solutions. In Pyrex beakers the drift was tolerably slow, but frequent readjustment is necessary, preferably before each set of experiments if the interval between experiments is more than 2 days.

Precaution has to be taken in the colorimetric evaluation of the toluidine blue solutions. The dye is not completely stable in acid methanol, and in the course of 1 week or more, a decrease in the absorption becomes noticeable even with cruder instruments. This change was not observed with aqueous solutions of the dye. Therefore, it was necessary to plot a calibration curve from a freshly prepared standard solution. In this study frequent checks with fresh standard solutions were carried out.

After constant use the surfaces of the slides acquire a yellowish appearance, and a substantial amount of dye is absorbed by these altered surfaces. It is advisable therefore to use new slides as soon as the surfaces show deterioration. In some instances the tissue was lost upon immersion in the dye solution. This phenomenon occurred rather irregularly and its cause was not ascertained, but could be eliminated by adding about 0.5 ml. of acetone to each slide before the homogenate was dried. Applying these precautions, we could reproduce our results on aliquots of the same homogenate within an error of 10 per cent. The homogenates could be diluted to one-fifth of the original concentration and proportionate dye uptake was obtained within the limits of error. Further dilution yielded too high values, possibly due to the increasing rôle of small amounts of dye adhering to the glass. Comparison of coarse and fine homogenates did not reveal noticeable deviations of the dye uptake.

Results

For the determination of the dye-binding capacity of muscle, 0.1 ml. aliquots of each individual homogenate were spread out on two or three separate slides which were run through the staining procedure and the

colorimetric determination, as outlined in the previous section. The average values for such duplicate or triplicate determinations furnish the individual figures in the samples in Table II. The scattering among samples from the same homogenate is not more than 10 per cent, while the figures for different homogenates from tissues of the same age vary over a much wider range. The ranges therefore listed in Table I indicate variations which are inherent in the tissue samples and do not represent error of method. The dry weight referred to in this table is the weight of the precipitate obtained from the homogenate with 5 per cent trichloroacetic acid, subsequent washing with alcohol, and drying at 90° overnight.

The data collected in Table I show that the dye-binding capacity of muscle tissue decreases sharply with increasing age, and also changes with

TABLE I

Amount of Toluidine Blue Bound by Muscle Tissue Homogenate at Different Stages of Development in Mg. of Dye per 100 Mg. of Tissue, Dry Weight

The data are the result of duplicate or triplicate runs for five different tissue samples for each age.

Age		pH 6.8	pH 4.2	pH 3.8	pH 3.2
13 days of gestation	Range	9.7-23.4	7.1-13.7	4.7-11.2	2.5-9.2
	Average	16.5	11.3	7.3	4.5
17 days of gestation	Range	9.4-18.3	5.1-11.8	4.9- 5.6	2.2-5.1
	Average	13.7	7.8	5.3	3.2
1 day after birth	Range	6.8-13.5	4.0- 5.5	2.1- 3.8	1.8-2.2
	Average	11.3	4.9	3.1	1.9
9 days after birth	Range	10.1-10.8	2.6- 4.5	0.8- 2.0	0.5-1.5
	Average	10.6	3.5	1.5	1.1

the pH values of the staining solution. However, there is a difference between the slopes for this drop with age for the different pH values. At pH 6.8 the lowest value, corresponding to an age of 9 to 10 days, is about 65 per cent of the highest value for the 13 day embryo. At pH 4.2 we have a drop to 31 per cent, at pH 3.8 to 21 per cent, and at pH 3.2 to 25 per cent.

In order to interpret these figures, it was necessary to define the tissue constituents involved in the combination with dye. From the literature cited in the introduction, it appears well established that the main fractions which take part in the combination with basic dyes are the proteins and the nucleic acids. An experiment, therefore, was planned to give information about the dye-binding characteristics of one of these components in the absence of the other. To accomplish this, the nucleic acids were removed from muscle tissue and the dye-binding capacity of the re-

maining material was determined. A complete extraction of nucleic acids can be accomplished, according to Schneider (15), by heating the tissue in 5 per cent trichloroacetic acid for 15 minutes to 90°. 200 mg. of muscle tissue from new-born rats, after homogenization, were extracted in 5 per cent trichloroacetic acid at 90°. The remaining tissue was washed in the centrifuge three times with 0.5 M KCl of pH 6.8 and three times with distilled water. The washing with KCl has the purpose of removing as much of the trichloroacetic acid as possible and the excess KCl is removed by the distilled water. The tissue was then suspended in 2 ml. of water and the dye-binding capacity was determined. By comparing the data in Table I for the new-born rat with the values obtained in this experiment, the differences due to the extraction of nucleic acid are readily recognized. At pH 6.8, a dye uptake of 6.4 mg. was obtained in the extracted tissue as against 11.3 mg. in the unextracted, while at pH 4.2 the respective values are 1.2 and 4.9. For lower pH values, the figures for the extracted tissue remain unchanged at about 1.2 mg. The comparison of the figures for extracted and for unextracted tissue suggests the following; in the absence of nucleic acids only a small and constant amount of dye is bound at pH 4.2, which remains unchanged on further lowering of the pH. A great increase in the dye binding occurs in extracted tissue above pH 4.2, suggesting that the carboxyl groups of proteins become available for the combination with dye. The excess of bound dye in unextracted tissue over that of extracted tissue indicates that the dye-binding capacity is due to nucleic acids. This amounts to 0.036 mM of dye at pH 4.2 and 0.049 mM of dye at pH 6.8. The amount of dye bound by the same amount of nucleic acid increases from pH 4.2 to 6.8 by about 26 per cent. A dependence of dye combination with the nucleic acids in the tissue on pH for the range pH 6.8 to 4.2 seems possible, since such a relation is also apparent for lower pH values decreasing to pH 3.2. While the extracted tissue which is free from nucleic acids shows only a small and constant dye uptake for the pH range 4.2 to 3.2, a marked decline of the dye uptake was observed in unextracted tissues which contain the nucleic acids (Table I).

The preceding experiments yield some information about the participation of nucleic acids and of proteins in the dye uptake at different pH values. As the next step, it seemed desirable to find out more about the factors which cause the pH dependence of the dye combination with nucleic acids. One alternative was the possibility that the lower dye binding with decreased pH was due to repression of the dissociation of the negative charges of the nucleic acids, presumably of their phosphate groups. The second was a change in the interaction between proteins and nucleic acids, leading to a decreased availability of the phosphate groups by interaction

with the positive charges of the proteins. Experiments were next performed to determine the pH dependence of the dye combination with pure nucleic acids.

Dye-Binding Capacity of Ribose Nucleic Acid—The dye-binding capacity of nucleic acid was tested by determining the ratio of nucleic acid to dye in precipitates which are formed on mixing solutions of these two substances. To 1.0 ml. of a 0.1 per cent solution of commercial sodium ribonucleate (Schwarz Laboratories, Inc., 9.05 per cent P) were added 9.0 ml. of a 0.001 M toluidine blue solution which was adjusted to the desired pH. After mixing, the pH was checked and readjusted if necessary, and the mixture was allowed to stand for several hours until a flocculent precipitate had formed. The precipitate was centrifuged off and washed in the centrifuge with ice-cold distilled water until the supernatant showed only a faint color. The precipitate was suspended in 10 ml. of a solution which contained 2.5 ml. of H_2SO_4 and 2.5 ml. of H_2O in 95 ml. of methanol and was centrifuged after stirring frequently for about 5 to 10 minutes. The precipitate showed only a faint coloration and practically the entire dye content was dissolved in methanol- H_2SO_4 . This extract was used for the colorimetric determination; the nucleic acid content was determined by phosphate determination. The precipitate was digested with 0.3 ml. of concentrated H_2SO_4 in a sand bath at 250–300° for about 5 hours, with addition of 1 to 2 small drops of H_2O_2 at the end of the digestion period. 3 ml. of water were added and the tubes were immersed for 10 minutes in a boiling water bath to hydrolyze pyrophosphates. The samples were neutralized, made up to a volume of 5 ml. with water, and the color developed according to Lohmann and Jendrassik (16), with purified amino naphtholsulfonic acid as the reducing agent. The readings were made in a Cenco-Sheard photometer at 660 $\mu\mu$. For the phosphorus determinations in the H_2SO_4 -methanol extracts, 5 ml. aliquots were used. The methanol was evaporated on a water bath and the water was removed by heating overnight in an oven at 110°. The H_2SO_4 present in these aliquots is sufficient for digestion, and the remaining procedure follows the description above. Phosphate determinations on the supernatant were carried out by evaporating the water after addition of H_2SO_4 in a quantity sufficient for the following digestion.

The results of this experiment are listed in Table II. The amount of dye in the precipitate which is completely extracted by the methanol- H_2SO_4 mixture remains rather constant for the pH range from pH 3.2 to 6.8. The calculation in mM is based on an actual dye content of the commercial product of 59 per cent, as indicated by the accompanying label. The greater part of the nucleic acid remains in the precipitate and is not extracted by the acid alcohol mixture; about one-fourth, however, is re-

covered in the extract. The total nucleic acid content of the precipitate is calculated as the sum of nucleic acid contents of extract and of the precipitate after extraction. The recovery totals nearly 80 γ of nucleic acid phosphate, which is 9.05 per cent of our nucleic acid preparation, giving thus 890 γ of nucleic acid. Since we used 1 mg. of nucleic acid for each sample, 89 per cent is recovered in the precipitate. We did not find a significant change in the amounts of nucleic acid phosphate when the precipitation was carried on at the different pH values. The ratio of dye to nucleic acid phosphate in the precipitate remains therefore constant over the range of pH values which were used in this experiment. Expressed in mM, the ratio is close to unity. This suggests that each phosphate group of the nucleic acid combines with 1 dye molecule, and that

TABLE II

Content of Dye and Nucleic Acid Phosphorus in Precipitate Obtained by Mixing Solutions of Toluidine Blue and Ribose Nucleic Acid

pH of mixture	Dye content of H ₂ SO ₄ -methanol extract		P content				(4, 5) + (6, 7)		Ratio, $\frac{(3)}{(9)}$
			Of ppt. after extraction		Of H ₂ SO ₄ -metha- nol extract				
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	
	γ	$\frac{mM}{\times 10^{-3}}$	γ	$\frac{mM}{\times 10^{-3}}$	γ	$\frac{mM}{\times 10^{-3}}$	γ	$\frac{mM}{\times 10^{-3}}$	
6.8	770	2.85	69	2.2	15	0.5	84	2.7	1.05
4.2	740	2.74	64	2.1	15	0.5	80	2.6	1.05
3.8	650	2.40	59	1.9	12	0.4	71	2.3	1.04
3.2	710	2.62	66	2.1	14	0.5	80	2.7	0.97

The figures are calculated for the whole precipitate obtained by mixing 1 ml. of 0.1 per cent ribose nucleic acid and 9 ml. of 0.001 mole of toluidine blue.

the process resembles a true salt formation. Since the combination of toluidine blue and nucleic acids in pure solutions does not seem to be dependent upon pH, a change in the dissociation of the negatively charged groups of nucleic acids cannot be held responsible for the pH dependence of dye combination of nucleic acids in tissues.

Combination of Toluidine Blue and Nucleic Acid in Presence of Fibrinogen—The lack of dependence of dye combination with pure nucleic acids made it seem desirable to simulate the conditions occurring in tissues where nucleic acid is mainly combined with protein; i.e. as nucleoprotein. Therefore, the dye was added to a solution which contained not only the nucleic acid but also a protein. This experiment was performed in two ways. In the first, 1 to 2 ml. of a 1 per cent solution of fibrinogen (Armour) were added in 0.5 mole of KCl to 1 ml. of a 0.1 per cent solution

of ribose nucleic acid in distilled water. This mixture did not show a distinct precipitation. After addition of 8.0 ml. of a 0.001 mole of toluidine blue solution, a precipitate developed which was allowed to stand for several hours. In all instances the toluidine blue solution was adjusted to the desired pH before addition, and the pH was readjusted after mixing. In another set of determinations, 5 ml. portions of the above solutions of nucleic acid and of fibrinogen were mixed and kept for 5 minutes in a boiling water bath. A precipitate formed which was dispersed after cooling with a glass homogenizer. To 2 ml. aliquots of this mixture were added 8 ml. of the toluidine blue solution, and the sample was allowed to stand for several hours. The precipitates were analyzed for the content

TABLE III

Content of Toluidine Blue and Nucleic Acid in Precipitate Obtained from Mixtures of Solutions of Fibrinogen, Nucleic Acid, and Toluidine Blue

Unheated series, mixture of fibrinogen and nucleic acid prepared at room temperature; heated series, mixture of fibrinogen and nucleic acid prepared in boiling water bath.

The figures indicate the average of two to six separate determinations in $\text{mm} \times 10^{-3}$.

Unheated				Heated		
pH	Dye content (1)	Nucleic acid P (2)	Ratio, $\frac{(1)}{(2)}$ (3)	Dye content (4)	Nucleic acid P (5)	Ratio, $\frac{(4)}{(5)}$ (6)
6.8	3.2	3.2	1.00	2.2	2.0	1.10
4.2	2.6	2.8	0.93	1.5	1.6	0.94
3.8	2.1	2.8	0.75	1.2	1.6	0.75
3.2	1.9	2.8	0.68	0.7	1.5	0.47

of dye and of nucleic acid phosphorus, as described above, with certain precautions. For complete removal of loosely bound or occluded dye it was necessary to wash the precipitate three times in the centrifuge with 10 ml. portions of ice-cold water of pH 6.8. The pH of the wash is not too critical if kept above pH 6, but at pH 3.2 about half of the dye is lost from the precipitate. Varying the time of washing from 1 to 3 hours did not show marked deviations, but extension of the washing over 24 hours decreased the dye content in the precipitate by about 20 per cent. When the precipitate was formed by heating, a variation of the heating time from 5 to 10 minutes was not significant.

The results of these experiments are presented in Table III. The distribution of dye and of nucleic acid in the precipitate, in the acid alcohol extract, and in the supernatant was similar to the results of the preceding

experiment. The data for the toluidine blue content in the precipitate again refer to the amount of dye extracted with alcohol, and the figures for the nucleic acid phosphorus represent the sum of the contents of the precipitate after acid alcohol extraction and of the acid alcohol extract itself. Since the results for different samples did not vary more than 8 per cent, we included in Table III only the averages of two to six separate determinations for each listed value.

The figures in Table III show a definite decrease for the ratio, toluidine blue to nucleic acid phosphorus, for the decrease in pH from 4.2 to 3.2. This decrease is about twice as great in the heated samples as in the unheated samples. The results obtained in this series differ markedly from

TABLE IV

Correlation of Nucleic Acid Content and Dye Binding in Rat Muscle Tissue at Different Stages of Development

Age	Nucleic acid P (1)	Amount of bound dye in mm per gm. dry weight† (2)				Ratio of dye bound to nucleic acid, Column 2 to Column 1			
		pH of staining solution				pH of staining solution			
		6.8	4.2	3.8	3.2	6.8	4.2	3.8	3.2
days	mm per gm., dry weight*								
13 (G.)	0.53	0.61	0.42	0.27	0.17	1.05	0.79	0.51	0.32
17 "	0.40	0.51	0.29	0.20	0.12	1.27	0.73	0.50	0.30
1 (B.)	0.21	0.42	0.18	0.11	0.07	2.00	0.86	0.52	0.33
9 "	0.15	0.39	0.13	0.06	0.04	2.68	0.89	0.41	0.25

G., age in days during gestation; B., days after birth.

* Calculated from the data in a previous paper (nucleic acid).

† Calculated from the data in Table I.

the previous data with mixtures of nucleic acid and toluidine blue in the absence of protein. The experiment suggests therefore that the decline in the dye combination of nucleic acids with decreasing pH is due to the presence of proteins. The difference in the results between heated and unheated tissue indicates furthermore that it is not merely the presence of the protein which is responsible for this phenomenon but the state of the protein which makes a close interaction between nucleic acids and proteins possible. At pH 4.2 and 6.8 the values for the ratio are close to 1, which agrees with the data in the previous experiment. The nucleic acid phosphorus in this series shows some decrease from pH 6.8 to 4.2 which cannot be explained at the present time, but it must mean that some nucleic acid is kept in solution at the lower pH values under the conditions of the present experiment.

DISCUSSION

The aim of the experiments reported in this paper was a quantitative interpretation of the combination of the basic dye, toluidine blue, with the nucleic acid and protein constituents of the developing muscle cells at different stages of development. The data are correlated in Table IV. The nucleic acid concentrations are calculated from previous data (13) and the toluidine blue content is calculated from Table I.

There are two main trends apparent in the amount of dye combined with developing muscle tissue; (1) the decrease with progressing development and (2) the decrease with decreasing pH. At pH values of 4.2, 3.8, and 3.2, the decline in the content of nucleic acid and dye with age is in close agreement, as indicated by the consistency in the ratios of bound dye to nucleic acid.

At pH 6.8 this ratio more than doubles from the 13th day, intrauterine, to the 10th day after birth. At pH 6.8, both nucleic acid and proteins participate in the dye binding, while, at pH 4.2 and lower, the dye interaction is due essentially to nucleic acids alone. The protein content increases with age (13) and the ratio of dye to nucleic acid increases at pH 6.8, since an increasing fraction of the dye is bound by protein. From the experiments with extracted tissue at pH 6.8, it is evident that nucleic acids combine with about 26 per cent more dye than at pH 4.2. By adding this percentage to the dye content at pH 4.2, one obtains an approximate figure for the amount of dye combined with nucleic acid at pH 6.8. The difference between this figure and the full value for the dye content at pH 6.8 is then an index for the amount of dye combined with proteins. In progressing order for the developmental stages, these figures are 0.08, 0.14, 0.19, and 0.23, corresponding to an increase by a factor of 2.9. It is known that protein dry weight in muscle tissue for this period increases by a factor of 2.2. This shows that the results from the two sets of data are of the same order of magnitude. If the staining is carried out at a controlled low pH (4.2), structures which are distinctly stained could be nucleic acids or other substances with stronger dissociation of anionic residues, while the carboxyl groups of proteins probably can be excluded from the reaction.

The decrease of the dye-combining power of tissues below pH 4.2 is apparently not attributable to changes in the nucleic acid molecule itself, since the combination of dye with pure nucleic acids is independent of the pH within the examined range. This decrease can be attributed to the interaction of nucleic acids with proteins since a similar decrease can be observed with mixtures of ribose nucleic acids with fibrinogen. The extent of this decrease seems to be dependent not only upon the quantity

of a protein but also upon its specific structure. In experiments with fibrinogen the pH sensitivity of the dye combination after admixture of the protein at room temperature was smaller than after boiling the protein in the presence of nucleic acids. The increase of the reactivity of protein groups on heat denaturation is a well known phenomenon, and it is likely that nucleic acids combine more firmly with fibrinogen if basic groups become available in the course of heating. The pH sensitivity of the dye combination at a pH value below 4.2 seems to be an indicator of the affinity and extent to which the groups of the nucleic acids responsible for the combination with dye are in combination with the basic groups of proteins.

SUMMARY

With the present day approach to the chemical embryology of cells and tissues it has become increasingly evident that the salt formation concept of basic dye interaction has definite limitations. The present study attempts to throw light on this problem by studying the combining power of the basic dye, toluidine blue, with chemical properties of developing rat muscle.

The nucleic acid content of developing rat muscle can be correlated with the dye-binding capacity at different pH values and maintains a strict relationship over a part of the pH range examined. For comparison, a commercial nucleic acid product and tissue from which the nucleic acid had been removed were similarly studied.

The amount of dye combining with developing muscle tissue decreases during development and also decreases with a lowered pH; at pH 4.2 the nucleic acid alone unites with the dye, while at pH 6.8 both the nucleic acid and proteins combine with it. The decrease in dye-combining power is not due to changes in the nucleic acid molecule but is attributable to the interaction of nucleic acids and proteins.

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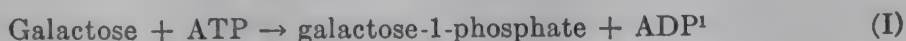
ISOLATION OF THE COENZYME OF THE GALACTOSE PHOSPHATE-GLUCOSE PHOSPHATE TRANSFORMATION*

By R. CAPUTTO, LUIS F. LELOIR, C. E. CARDINI, AND A. C. PALADINI

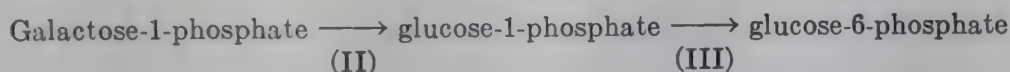
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The first step in the utilization of galactose has been found to be a phosphorylation by adenosine triphosphate (ATP) catalyzed by galactokinase (1-3).



Evidence has been presented (4) showing that the enzymatic transformations which follow are



In the initial studies (5) it was found that a thermostable factor was required in the over-all Reactions II and III. This fact led eventually to the discovery and isolation of glucose diphosphate (6), which acts as a coenzyme in Reaction III. These results have been confirmed by Sutherland *et al.* (7), and biological (8, 9) as well as chemical (10, 11) methods for the synthesis of glucose diphosphate have been described.

Reaction II, which consists in a Walden inversion of C-4, is catalyzed by an enzyme which is currently called "galactowaldenase" in this laboratory. The corresponding coenzyme has been named uridine-diphosphate-glucose (UDPG) for reasons which will become apparent in what follows.

Estimation and Isolation of UDPG

The method of estimation of UDPG was based on its property of accelerating Reaction II. In practice the rate of Reaction II was measured as the sum of Reactions II and III by the rate of appearance of reducing power. The reaction mixture contained galactose-1-phosphate and a maceration juice of *Saccharomyces fragilis* plus variable amounts of UDPG. Glucose diphosphate was also added; so that the velocity of the over-all reaction was limited by the rate of Reaction II. The results obtained by varying the amounts of UDPG are shown in Fig. 1.

* A preliminary note has been published (*Nature*, **165**, 191 (1950)).

¹ Adenosine diphosphate.

Of the different sources of coenzyme tested as starting material for the purification, bakers' yeast was found to be the best, but it is of interest that animal tissues were also found to contain UDPG, although in slightly smaller amounts. The yeast used was not adapted to galactose and hardly fermented this sugar. This raises the question as to whether UDPG may not have some other function besides accelerating the galactose-glucose transformation.

The UDPG content of yeast was found to be increased for unknown reasons after a short incubation with toluene. The amount became at least doubled, and use of this fact was made in the large scale preparation.

Purification of UDPG was effected by extraction of the toluene-treated yeast with 50 per cent ethanol followed by precipitation with mercuric salts. The mercury precipitate was then suspended in 1 M ammonium acetate when most of the UDPG was dissolved, leaving a considerable

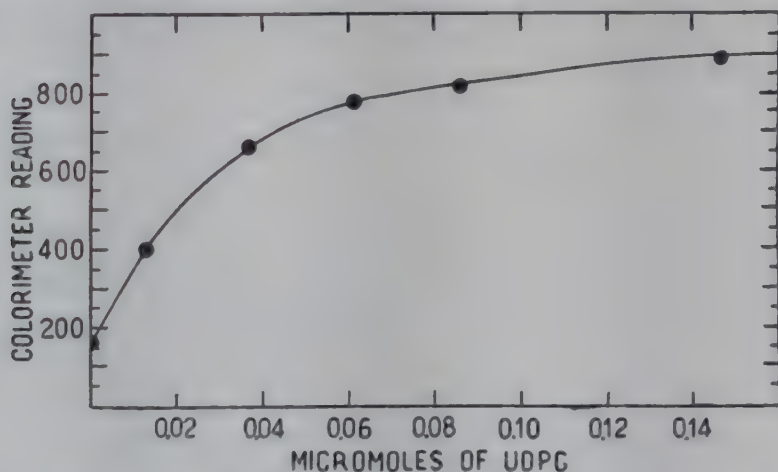


FIG. 1. The activation of galactowaldenase by UDPG

amount of impurities which were insoluble. After reprecipitation the precipitate was decomposed with H_2S in the cold. The next step was adsorption on charcoal and elution with 50 per cent ethanol. Amino acids were then removed with a cation exchange resin and the charcoal adsorption repeated.

The product obtained had maximum absorbency at $260\text{ m}\mu$. Many other procedures of purification were applied, but it was not possible to increase the ratio of activity-absorbency at $260\text{ m}\mu$. This was an indication that the absorption was due to the active substance. Confirmation of this fact was obtained by paper chromatography which showed that the activity always ran parallel with the absorbency at $260\text{ m}\mu$.

Presence of Uridine

The absorption spectrum of UDPG at different pH values is shown in Fig. 2. The curves agree well with those of uridine shown in the same

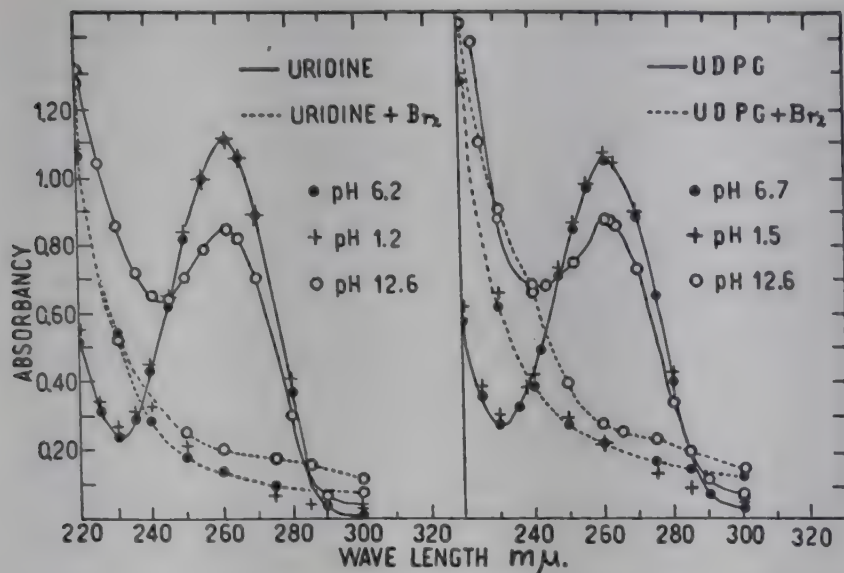


FIG. 2. Comparison of the spectra of UDPG and uridine at different pH values and after bromine treatment. The latter was carried out in acid reaction by addition of a drop of bromine water followed by aeration.

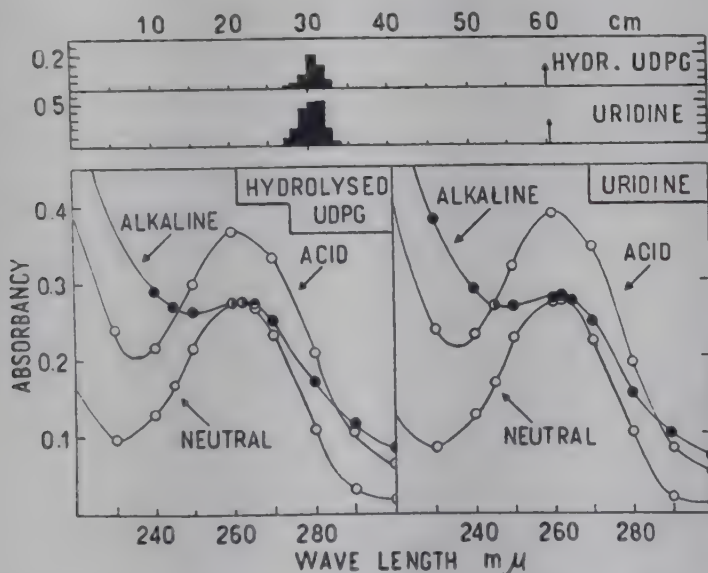


FIG. 3. Comparison of a known sample of uridine with the nucleoside obtained by hydrolysis of UDPG. The upper figure shows the position of the substances as revealed by the absorbency at 260 μ m after paper chromatography with 77 per cent ethanol. Hydrolysis of UDPG was carried out for 40 hours in 0.1 N sulfuric acid, followed by removal of the acid with barium hydroxide and concentration. The chromatogram was developed according to the procedure of Hotchkiss (27). The lower figure shows the spectra of the substances extracted from the paper after the chromatography. These spectra are not exactly equal to those of non-chromatographed uridine. The difference could be produced by the action of light and probably took place during the drying of the papers.

figure and are clearly different from those of other purine or pyrimidine derivatives (12). The effect of bromine, which destroys completely the coenzymatic activity, is also shown.

As shown in Fig. 3, uridine could be identified after acid hydrolysis by comparison with a known sample with respect to behavior during paper chromatography and to the spectra at different pH values.

Uracil could also be identified by chromatography and by its spectrum after hydrolyzing UDPG 6 hours in 2.5 N sulfuric acid at 100°. Moreover, it was not precipitated by silver salts under the conditions in which purine bases are completely precipitated (13).

Estimation of the pentose in the hydrolysis products of UDPG carried out by the orcinol method after bromination as described by Massart and Hoste (14) gave the expected values. It was observed that the rate of development of the color was more rapid than with free ribose or uridine.

Presence of Phosphate

Once uridine was proved to be a constituent of UDPG, it was possible to calculate the concentration of the solutions by means of the molar absorbency index of uridine. This constant has been measured by Ploeser and Loring (12), who gave the value $A_m = 9820$. The value for uridylic acid was practically the same (10,040). Estimations of phosphate in UDPG revealed 2 molecules per molecule of uridine. Conversely, calculation of the molar absorbency on the assumption of two phosphates gave a value of 10,450. One of the phosphate groups is acid-labile and can be hydrolyzed in 15 minutes in 1 N acid at 100°. The hydrolysis curves for the labile phosphate are shown in Fig. 4. Comparison with other known compounds reveals that the rate of hydrolysis is slower than that of the labile phosphate of adenosine triphosphate and of about the same order as that of adenosine-3'-phosphate.

The rate of hydrolysis of the stable phosphate in 0.1 N acid gave values comparable with those given by Gulland and Smith (15) for uridine-2'- and 5'-phosphates, but nearer the former. The rate was definitely lower than that given for uridine-3'-phosphate.

Presence of Glucose

Besides uridine and two phosphate groups, UDPG was found to contain a reducing substance which could be split off by mild acid hydrolysis. The results of the carbazole reaction (16) on UDPG did not give clear cut results, but after acid hydrolysis and removal of contaminants with ion exchange resins, the color corresponded to that given by glucose. Moreover, the sugar was found to be fermented by *Candida monosa*, which does not act on galactose.

Paper chromatography with 77 per cent ethanol, by which galactose can be distinguished from glucose, also indicated the latter as the component of UDPG.

The rate of liberation of glucose from UDPG on acid hydrolysis is shown in Fig. 5. This rate is about 6 times higher than that of glucose-1-phos-

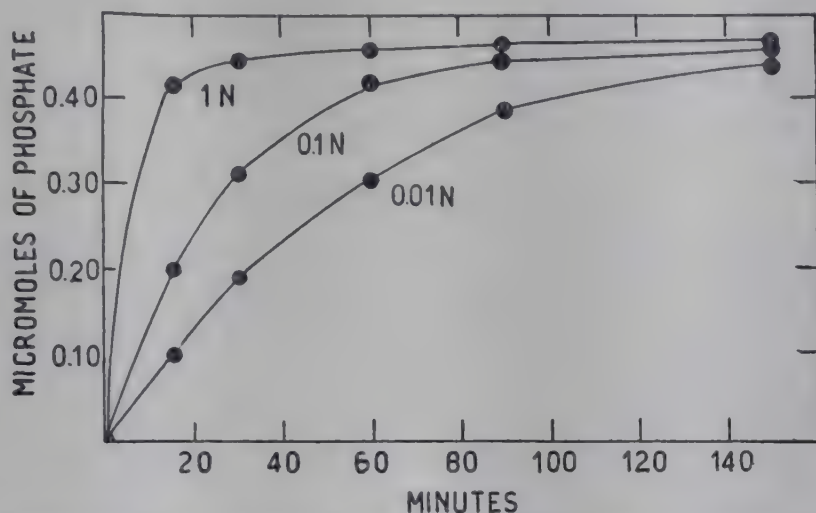


FIG. 4. Hydrolysis of the labile phosphate of UDPG in acid at 100°. Total phosphate of samples, 0.9 μ M.

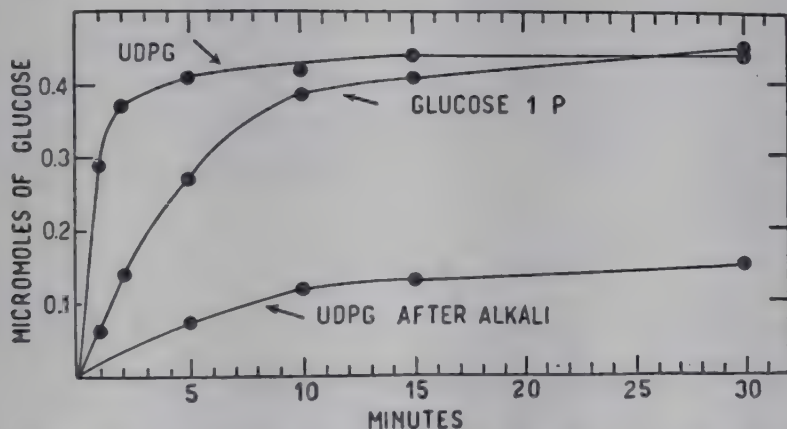


FIG. 5. Liberation of glucose in 0.01 N acid at 100°. Upper curve, untreated UDPG. Lower curve, UDPG which had been heated 30 minutes at 100° in 0.01 N sodium hydroxide. Glucose-1-phosphate is shown for comparison.

phate and about 40 times higher than that of the labile phosphate of UDPG. These differences in rates of hydrolysis allowed the isolation of a uridine diphosphate and a uridine monophosphate from UDPG.

That the bound glucose was part of the active molecule was proved by the constancy of the ratio uridine-phosphate-glucose, which always remained 1:2:1 after various fractionation procedures. Additional evidence on this point was obtained by comparing the rate of liberation of glucose

with the rate of inactivation of the coenzyme in 0.01 N acid at 100°. The results were as follows:

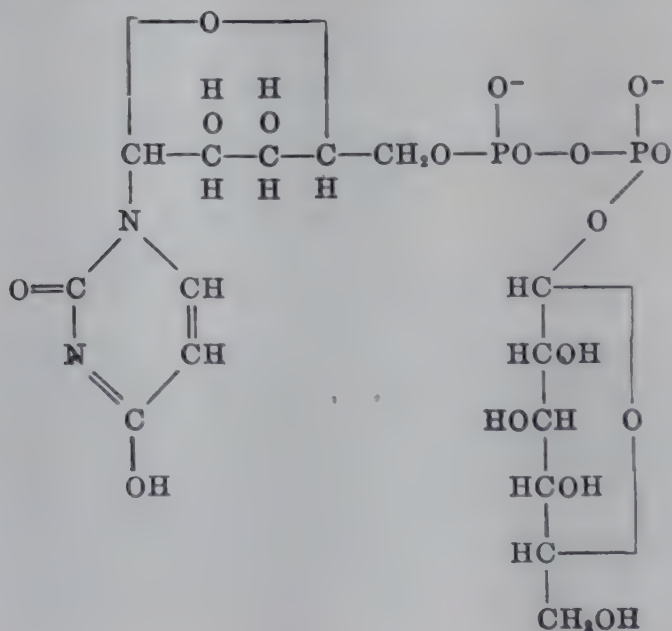
Time of heating, min.....	1	2	5
% glucose liberated.....	64	82	91
" inactivation.....	50	75	100

Oxidation of UDPG with periodate gave 1 molecule of formic acid, as if positions 2, 3, and 4 of the glucose were free.

Linkage of Different Components

Uridine, two phosphate groups, and glucose having been identified as components of UDPG, there remained to find out how they are combined in UDPG and whether or not there is some other component. Nitrogen estimations gave 2 atoms per molecule; thus any other nitrogen-containing substance besides uridine could be excluded. Dry weight measurement gave a molecular weight of 630, calculated from the uridine content. The theoretical weight for the sum of uridine, glucose, and two phosphate groups minus 3 molecules of water is 566. The preparation would thus be 90 per cent pure.

Electrometric titration as shown in Fig. 6 indicated two acid groups in the range of the primary dissociation constant of phosphoric acid and no secondary. On treatment with acid secondary phosphoric acid groups appeared: one after hydrolysis of the glucose and another after the labile phosphate became inorganic. The accompanying formula agrees well with these findings.



The constant of hydrolysis of the labile phosphate in UDPG is about 10 times slower than that of ATP. This fact cannot be interpreted against the pyrophosphate structure of UDPG, because uridine phosphates are more stable than the corresponding adenine phosphates. The approximate values of the constants of hydrolysis are shown in Table I.

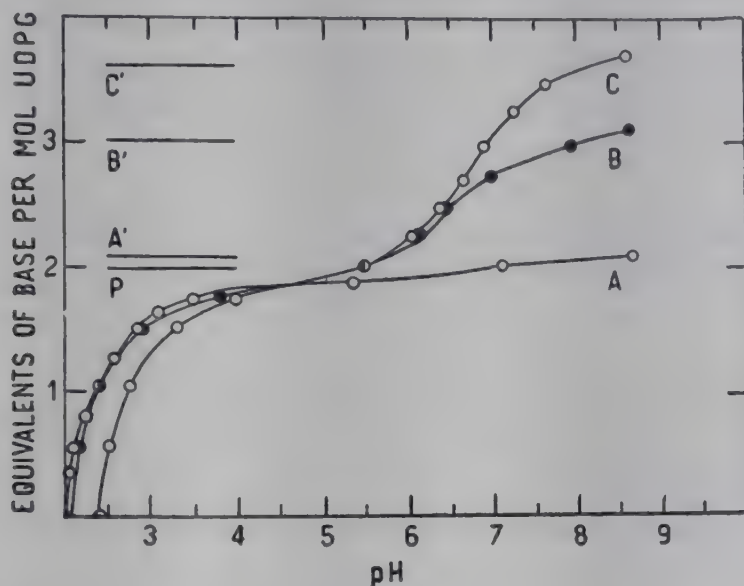


FIG. 6. Electrometric titration of UDPG before and after hydrolysis. Sample A, 14.7 μ M of unhydrolyzed UDPG. Sample B, after heating the free acid 4 minutes at 100°. Sample C, after 60 minutes under the same conditions. The analytical results in micromoles were as follows:

	Sample A	Sample B	Sample C
Total phosphate (P_T).....	29.52	29.52	29.5
Inorganic phosphate (P_0).....	0.48	2.40	9.92
Free glucose (G).....	0.84	12.48	14.04
$P_T + P_0 + G$	30.84	44.40	53.48

Levels A', B', and C' correspond to the value of $(P_T + P_0 + G)/UDPG$. Level P corresponds to $P_T/UDPG$. See the text.

Position of Phosphate Group in Uridine

The phosphate seems to be attached to position 5 of the pentose in uridine. One test which was applied is based on the property of substances containing neighboring hydroxyls to form a complex with copper. Klimeck and Parnas (17) observed that adenosine-5'-phosphate forms such a complex, whereas adenosine-3'-phosphate does not. Since this test requires considerable amounts of substance, it was adapted to a microscale by estimating the copper with diethyl dithiocarbamate, as described by Stiff (18) for the estimation of protein. Comparative tests with adenosine-3'-

and 5'-phosphates, uridine-3'-phosphate, and uridine monophosphate prepared from UDPG showed that the latter behaved as if the phosphate were in position 5' (Table II).

Another test which was carried out was the measurement of the rate of color development with the orcinol reagent. This rate was found by Albaum and Umbreit (19) to depend on the position of the phosphate group in ribose derivatives. It is higher for ribose-5-phosphate than for ribose-3-phosphate or free ribose and the same is true for the corresponding adenylic acids. This test cannot be applied directly to uridylic acid due to the

TABLE I

Hydrolysis Constants (Log₁₀) for Phosphate of Some Nucleotides
The figures in parentheses refer to the bibliography.

	K
Labile phosphate of UDPG.....	0.0039
“ “ “ ATP.....	0.038
Adenosine-3'-phosphate (33)	0.0036
Uridine-3'-phosphate (15)	0.0005
Adenosine-5'-phosphate (33)	0.00034
Uridine-5'-phosphate (15)	0.00008

TABLE II

Copper Complex Formation

	Colorimeter reading*
Adenosine-5'-phosphate, 2 μM	600
Adenosine-3'-phosphate, 2 μM	58
Uridine-3'-phosphate, 2 μM	64
Uridine monophosphate from UDPG, 2 μM.....	610

* Klett-Summerson photocolormeter with Filter 44.

stability of the ribose-uracil linkage. However, the uracil nucleus can be destroyed by treatment with hydrazine (20) followed by nitrite, and then the ribose phosphate can be isolated.

As shown in Table III, the rate of color development with the orcinol reagent of the ribose phosphate isolated from UDPG follows closely that of adenosine-5'-phosphate and is different from that of free ribose or adenosine-3'-phosphate.

The only result which did not agree completely with the properties of uridine-5'-phosphate was the hydrolysis with acid. The uridine monophosphate from UDPG in 0.1 N sulfuric acid at 100° gave 18.3 and 33 per

cent liberation of phosphate after 7.42 and 21.2 hours respectively. Gulland and Smith (15) give 8.6 and 20.5 per cent for uridine-5'-phosphate, 12.5 and 29.5 for the 2'-phosphate, and 38 and 69 for the 3'-phosphate. The rate of hydrolysis was therefore more similar to that of uridine-2'-phosphate. However, there might have been some difference in the experimental procedures.

Action of Alkali

Heating with 0.01 N alkali destroys the coenzymatic activity in a few minutes. This fact does not appear to be readily explained by the above formula, since glucose-1-phosphate, the pyrophosphate group, and uridylic acid are known to be stable to alkali.

A study of the action of alkali revealed that it does not lead to the liberation of phosphate or to a permanent change in the absorption spectrum.

TABLE III

Color Development with Orcinol Reagent

Procedure as described by Albaum and Umbreit (19) but at 85°. Values in per cent of maximum.

Time, min.....	7	15	35	70	85	95
Ribose.....	8.9	28.3	66	94	98.1	100
Adenosine-3'-phosphate.....	5.9	23.6	64.2	94.5	97.9	100
Adenosine-5'-phosphate.....	42.6	70.1	91.4	99.1	100	100
Ribose phosphate from UDPG.....	42.4	69	91.2	99.2	99.6	100

The only change which could be detected is a change in the rate of liberation of glucose by acid. This change is shown in Fig. 5. It is not due to an alteration on the glucose residue, since glucose can be recovered unchanged after hydrolysis with stronger acid. After alkaline treatment the glucose is removed in normal acid at about the same rate as the labile phosphate, whereas in untreated UDPG the glucose is removed much faster than the phosphate. Moreover, after the action of alkali the titration curve shows the presence of one secondary acid group besides the two primary groups present in untreated UDPG.

An explanation of these changes would be a migration of the glucose residue from the phosphate to the pentose.

Relationship with Substance Accumulating in Staphylococcus aureus Treated with Penicillin

Park and Johnson (21) reported the accumulation of a substance in *Staphylococcus aureus* cells when grown in the presence of penicillin. This

substance contains uracil, a reducing substance, and two phosphate groups which hydrolyze at about the same rate as in UDPG. However, other properties appear to be different, since the sugar does not appear to be glucose and the nitrogen values were higher.

Due to the similarity of the substances some experiments were carried out with the compound from *S. aureus*. By means of the procedure described by Park and Johnson (21) a partially purified substance containing uracil, labile phosphate, and a reducing substance in the relation 1.4:1:0.7 was obtained. The tests did not reveal any activating or inhibiting action on the glucose phosphate $\rightarrow$ galactose phosphate transformation. Moreover, preliminary tests agreed with the work of Park and Johnson in relation to the non-identity of the carbohydrate with glucose.

EXPERIMENTAL

Methods

The methods were as described in previous papers (1, 2, 6). Galactose phosphate was synthesized according to the procedure of Colowick (22) and Kosterlitz (23). Dry weight measurements were carried out, after drying over phosphorus pentoxide at 56° for 3 hours, on acid UDPG prepared by treating the sodium salt with Amberlite IR-100. Nitrogen estimations were carried out by the Kjeldahl method.

Enzymatic Estimation

S. fragilis was obtained as described by Caputto *et al.* (1), spread out on plates in a layer 2 to 3 mm. thick, and allowed to dry in air at room temperature. The dry yeast was extracted with 3 volumes of 2.2 per cent diammonium phosphate at 5° for 24 hours. These extracts could be stored frozen for many months. The procedure for the estimation of UDPG was as described by Cardini *et al.* (6) for glucose diphosphate but with use of a reaction mixture which contained 2 μM of galactose-1-phosphate, 2 μM of Mg^{++} , 0.01 μM of glucose diphosphate, 0.01 ml. of undiluted *S. fragilis* extract, and UDPG in amounts ranging from 0.01 to 0.1 μM , in a total volume of 0.2 ml. The reaction was allowed to proceed 20 minutes at 37° and was stopped by addition of the copper reagent.

Distribution

Estimations were carried out on the extracts obtained by the addition of 1 volume of ethanol to the fresh organs, heating to boiling, filtering, neutralizing, and evaporating off the alcohol. Due to their glucose content, the liver extracts had to be purified by precipitation with excess mercuric salts as described in "Preparation."

The results in micromoles of UDPG per gm. of fresh tissue ranged be-

tween 0.2 and 0.3 for rat kidney, brain, and muscle. For the liver the values were 0.1 to 0.2 μM . In yeast the content was somewhat variable. In bakers' yeast the content was 0.4 to 1.0 μM . After 4 to 6 days at 5° the content was the same or higher in some cases. In brewers' yeast the amount was about 0.5 μM per gm.

Action of Toluene

It was found that a treatment of yeast with toluene increased the yield of UDPG. When yeast intimately mixed with 10 per cent of its weight of toluene is incubated at 35–37°, there occurs an increase in UDPG, which attains a maximum after about 40 minutes, followed by a slow decrease. For instance, one experiment gave the following changes.

Time of incubation, <i>min.</i>	0	10	20	30	40	60
UDPG concentration, μM per gm.....	0.6	1.7	1.9	2.0	2.1	1.8

The incubation in the absence of toluene produced no changes. The increases produced by toluene in different experiments varied from 50 to 400 per cent, and the maximum concentration attained never exceeded 2.5 μM per gm. of yeast.

Preparation

Step 1. Extraction—5 kilos of bakers' yeast were spread out in a layer about 5 cm. deep and heated in an incubator. When the temperature attained 35–36° the yeast was intimately mixed with 500 ml. of warm toluene. After 40 minutes at 35–36°, 5 liters of 95 per cent ethanol were added and the mixture heated in a water bath until it boiled. On the following day it was filtered through a 32 cm. Büchner funnel with a filter aid.

Step 2. Precipitation with Mercuric Salts—The filtrate was acidified with 5 N nitric acid until acid to Congo red paper. Then 30 ml. of mercuric acetate per liter were added. After mixing, the preparation was left overnight in the ice box. The suspension was filtered through a Büchner funnel until the precipitate was nearly dry. This precipitate was then blended in 600 ml. of 1 M ammonium acetate and left at room temperature for 2 hours. The suspension was filtered and the filtrate was acidified to Congo red paper with nitric acid, 30 ml. of mercuric acetate and 1 volume of ethanol were added, and the mixture left overnight in the cold. After filtration the precipitate was blended in 600 ml. of water and decomposed with hydrogen sulfide in the cold. The mercuric sulfide was filtered off, washed with 100 ml. of water, and the combined filtrates aerated and neutralized to pH 7. The results are shown in Table IV.

Remarks—The mercuric acetate solution was prepared by mixing 13.5

gm. of yellow mercuric oxide, 9.2 ml. of glacial acetic acid, and water to make 100 ml.

The optimum amount of mercury reagent to be added was ascertained in preliminary trials and was sufficient to precipitate nearly all the activity. Addition of more mercuric reagent gave a precipitate of inactive substances.

The extraction of the mercuric precipitate with ammonium acetate was also studied in small scale trials. A second extraction removed more activity from the precipitate, but the purity was inferior.

The decomposition with hydrogen sulfide was carried out in an ice bath and was complete in about 4 hours. The step is rather critical, since the active substance is very sensitive to acid.

TABLE IV
Results of Purification Procedure

Step No.		Volume of extract	Concentration of UDPG*	UDPG-total P ratio	Ratio, UDPG-absorbency at 260 m μ
		ml.	μM per ml.		
1	Yeast extract	11,000	0.65	0.072	0.016
2	After Hg ⁺⁺ and H ₂ S	1,500	4	0.375	0.043
3	1st charcoal elution	140	25	0.62	0.105
4	After resin	320	11	0.73	0.125
	3rd fraction†	14.4	7.3	0.51	0.107
	4th "	12	26	0.57	0.104
5	2nd charcoal eluates	12.6	30	0.40	0.101
	6th "	10.5	10	0.36	0.081
	7th "	16.2	6.5	0.41	0.096

* Measured by enzymatic method.

† First and second fractions discarded.

Step 3. Purification with Charcoal—Two lots of extract prepared as described were mixed. 1 ml. portions were then treated with charcoal to find the amount necessary for 90 per cent adsorption of the material with absorbency at 260 m μ . This corresponded to nearly complete adsorption of the active substance. In the preparation described 103 gm. of Pfanstiehl's norit A were used for the total amount of extract. After 15 minutes at room temperature with occasional shaking the suspension was filtered. The charcoal was washed with 300 ml. of water and then suspended in 400 ml. of 50 per cent ethanol. After 15 minutes the suspension was filtered and the filtrate concentrated at about 45° under reduced pressure to one-third of its original volume.

Remarks—A second eluate of the charcoal still contained activity but the

purity was inferior. Contaminating substances such as glucose diphosphate are not adsorbed by charcoal, while others like diphosphopyridine nucleotide are adsorbed but not eluted appreciably with aqueous ethanol.

At this stage the substance is nearly pure as judged by the ratio of activity to absorbency at 260 $m\mu$, but it still contains considerable amounts of substances giving a positive ninhydrin reaction.

Step 4. Treatment with Cation Exchange Resin—The extract from Step 3 was cooled, acidified to about pH 1.2 with hydrochloric acid, and treated five times with 15 gm. of moist Ionac C-200. The temperature was kept at 4–5° and the time of contact was 15 minutes each time. The liquid was then neutralized.

The amount of resin was that necessary to reduce the ninhydrin reaction by about 90 per cent.

Step 5. Second Adsorption with Charcoal—The liquid was treated with 26 gm. of norit. The necessary amount was ascertained as previously described. The suspension was filtered and washed with 100 ml. of water. It was then eluted by adding 50 per cent ethanol to the Büchner funnel which had been used for filtering and collecting the percolate in 10 to 15 ml. fractions.

The results of a preparation which was not one of the best are shown in Table IV.

Solubility of Different Salts

Basic lead acetate will precipitate UDPG even from very dilute solutions. With mercuric acetate in acetic acid solution it does not precipitate, but by addition of nitric acid to about pH 4 and 1 volume of ethanol complete precipitation is achieved.

The barium salt is very soluble in water. At pH 8 and after adding 2 volumes of 95 per cent ethanol the supernatant contains about 1.5 μM per ml. of UDPG. The sodium, potassium, and ammonium salts are very soluble in water. Tests with the brucine salt did not give a crystalline substance.

Correlation of Activity with Absorbency at 260 $m\mu$

A 0.05 ml. sample containing 1.2 μM of a pure preparation was deposited on No. 1 Whatman paper and chromatographed with 77 per cent ethanol (24) by the ascending technique (25, 26). After 16 hours the paper was dried and extracted according to the procedure of Hotchkiss (27). Bands of 5 mm. were immersed in 3 ml. of water for 2 to 3 hours. The absorbency at 260 $m\mu$ was measured and then the coenzymatic activity. For the latter 1 ml. of extract was used and, owing to the dilution, the amount of enzyme and the time of incubation were doubled. The results showed a complete

coincidence between absorbency and coenzymatic activity. The R_F value was 0.44.

Impure preparations chromatographed as described and developed with ninhydrin showed several spots, but they differed in position from the active substance. Other solvents such as butanol-water with or without acetic acid hardly produced any migration of UDPG. With phenol-water the migration was satisfactory, but the solvent is difficult to eliminate and interferes with the spectral analysis.

Estimation of Pentose

Direct estimation of pentose by the orcinol method of Meijbaum (28) gave hardly any reaction, as would be expected from a pyrimidine nucleotide. However, after treatment with bromine, as described by Massart and Hoste (14), better results were obtained. With intact UDPG a green precipitate appeared. If UDPG was hydrolyzed 20 minutes in 1 N acid at 100° and then adsorbed on anion exchange resin and eluted with normal acid, then the presence of pentose was clearly detected. On comparison with a uridine solution of equal absorbency at 260 m μ after a 10 minute heating period, the ribose content was 30 per cent too high. If the heating time was prolonged to 30 or 40 minutes, then the ribose content was equal to that of uridine. The faster rate of color development is probably due to the presence of the phosphoric acid group.

Identification of Glucose

For all the tests UDPG was hydrolyzed 5 minutes in 0.01 N acid at 100°. The liquid was then treated with both an anion exchange and a cation exchange resin (Ionac).

Paper chromatography was carried out with 77 per cent ethanol, the unknown being run between known samples of glucose and galactose. In every case the sugar from UDPG moved exactly like glucose ($R_F = 0.50$). The position of the galactose spot was clearly different ($R_F = 0.47$).

The carbazole reaction as described by Gurin and Hood (16) was also carried out comparatively with different sugars. The ratios of absorbency at 520 to 420 m μ found were as follows: sugar from UDPG, 2.1; galactose, 1.5; glucose, 2.0. If the carbazole reaction was carried out directly on UDPG, the color obtained was not typical for glucose.

Fermentation tests were carried out with *C. monosa*, which does not ferment galactose, and with *S. fragilis*, which does. Known samples of glucose and galactose were run at the same time, and after fermentation and centrifugation the sugars were estimated with the Somogyi reagent (29). The sugar from UDPG behaved like glucose.

Periodate Oxidation

The procedure described by Halsall, Hirst, and Jones (30) was used. Formaldehyde was detected with chromotropic acid (31). Formic acid was detected as described by Grant (32) and measured volumetrically.

The concentration of the UDPG solutions was estimated from the glucose liberated with 0.01 N acid at 100° for 5 minutes. The action of periodate on UDPG was slow and the formic acid content of the mixture reached a limit only after 24 hours. At this point the formic acid corresponded in several experiments to 0.8 to 1.0 molecule of formic acid per molecule of UDPG. No formaldehyde was detectable.

Preparation of Uridine Diphosphate from UDPG

As can be seen in Figs. 4 and 5, boiling in 0.01 N acid for 5 minutes liberates practically all the glucose and only a very small portion of the phosphate. After such a treatment it was possible to precipitate uridine diphosphate by addition of barium acetate at pH 8 and 1 volume of alcohol. The barium salt thus obtained was free of glucose.

Preparation of Uridine Monophosphate from UDPG

Inspection of Fig. 4 shows that in order to remove the labile phosphate completely it is necessary to heat to 100° in 0.1 N acid for about 90 minutes. In order to isolate uridine monophosphate, barium hydroxide was added to pH 9 and the precipitate of barium phosphate was washed and discarded. The solution plus the wash water was then treated with sufficient alcohol to precipitate all the organic phosphate. Usually about 12 volumes were necessary. The barium salt of uridine monophosphate thus obtained was free of reducing substances and inorganic phosphate.

Isolation of Pentose Phosphate from UDPG

The destruction of the uracil nucleus was effected by an adaptation of the procedure of Levene and Bass (20), as follows: treatment of UDPG with hydrazine hydrate; destruction of the excess reagents and the urea moiety with nitrite; precipitation of the ribose-containing compound as the barium salt, followed by acid hydrolysis and isolation of barium ribose phosphate.

An experiment was carried out as follows: A mixture containing 31 μM of UDPG in 1 ml. of water plus 0.14 ml. of hydrazine hydrate was heated 90 minutes at 65°. After cooling, 0.7 ml. of 20 per cent sodium nitrite was added and adjusted to pH 1.2 with hydrochloric acid. After a few minutes saturated barium hydroxide was added to pH 8, followed by 5 volumes of 95 per cent ethanol. The suspension was left overnight in the

ice box and then centrifuged. The precipitate was dissolved in 3 ml. of water and the barium removed with sodium sulfate. The liquid was then made 1 N with hydrochloric acid, heated 10 minutes at 100°, and then neutralized. Excess barium chloride was added and after several hours in the cold the precipitate was discarded. The liquid was then evaporated to dryness. The residue was dissolved in water and precipitated with several volumes of acetone.

The product obtained gave a relation of pentose to organic phosphate of 1:1.1.

Copper Complex Formation

The procedure described by Klimeck and Parnas (17) for distinguishing adenosine-3'-phosphate from adenosine-5'-phosphate consists in the addition of a copper salt and sodium hydroxide. When two neighboring hydroxyl groups are present as in the 5'-isomer, the supernatant appears blue.

By applying approximately the procedure described by Stiff (18) for measuring the copper complex formed by proteins, it was possible to carry out a reaction similar to that of Klimeck and Parnas but with 20 times less material. The procedure was as follows: To 0.5 ml. of the sample, about 3 mg. of copper phosphate and 0.5 ml. of 20 per cent trisodium phosphate were added. The suspension was intimately mixed with a glass rod. After 1.5 hours with occasional mixing the suspension was centrifuged. The supernatant was mixed with 1 ml. of 0.5 per cent diethyl dithiocarbamate and then extracted with 6 ml. of amyl alcohol. After centrifugation the absorbency of the alcohol layer was measured with a Klett-Summerson photocolormeter and Filter 44. The results are shown in Table II.

Electrometric Titration

The preparation of the free acid by decomposition of heavy metal salts of UDPG was not successful. The use of a cation exchange resin proved to be more convenient. The yield was about 90 per cent of UDPG, of which 94 to 98 per cent was the free acid.

In a typical experiment a 10 cm. column containing 3.5 ml. of Ionac C-200 which had been treated with 0.5 N hydrochloric acid and thoroughly washed with water was prepared. A solution of UDPG (55 μ M in 2.2 ml. of water) was passed six times through the column. The latter was then washed with water, making the final volume 10 ml. The whole operation was carried out at 5° and in about 5 minutes.

The solution was then divided into three samples. One (Sample A) was titrated as soon as it reached room temperature (15°). Samples B and C were titrated after being immersed in a boiling water bath for 4 and 60 minutes respectively.

After the electrometric titration with a glass electrode to pH 8.6, each sample was analyzed for inorganic and total phosphate and glucose, and the absorbency at $260\text{ m}\mu$ was measured. From the latter the concentration of UDPG was calculated. The results are shown in the legend to Fig. 6.

Of all the possible formulas which were tested the only one which explains the results is that shown above containing a pyrophosphate group.

According to this formula, UDPG should give two acid groups titrating to about pH 5, like the primary groups of phosphoric acid.

Curve A, Fig. 6, shows that this was the case. The small deficit in titratable groups in relation to total phosphate (about 6 per cent) may be attributed to incomplete elimination of cations. Secondary phosphoric acid groups which titrate between pH 5 and 8.6 were clearly absent.

According to the above formula, removal of the glucose should unmask one secondary phosphoric acid group. Subsequent liberation of inorganic phosphate should give one secondary and one tertiary acid group. Thus in partially hydrolyzed UDPG the primary plus secondary acid groups should be equal to the sum (in moles) of total phosphate plus inorganic phosphate plus free glucose.

The results shown in Fig. 6 agree with these predictions, as proved by the coincidence of the calculated Levels A', B', and C' with the amount of base necessary to titrate Samples A, B, and C to pH 8.6.

SUMMARY

The coenzyme of the galactose-1-phosphate $\rightarrow$ glucose-1-phosphate transformation has been studied. Methods are described for its estimation and isolation from bakers' yeast. The substance contains uridine, two phosphate groups, and glucose, and has therefore been named uridine-diphosphate-glucose (UDPG). As this coenzyme has been found to be present in animal tissues and in yeast not adapted to galactose, it is suggested that it may have some other function besides being the coenzyme of galactowaldenase.

Boiling in 0.01 N acid liberates practically all the glucose from UDPG in about 5 minutes and destroys the coenzymatic activity. Half of the phosphate in UDPG can be hydrolyzed in 1 N acid in 15 minutes at 100° .

The electrometric titration curve shows two primary phosphoric acid groups and no secondary. One secondary group appears on hydrolysis of the glucose and another on hydrolysis of the labile phosphate.

Experimental evidence is given which indicates that the uridine monophosphate obtained by hydrolysis of UDPG is uridine-5'-phosphate. A uridine diphosphate and the ribose phosphate could also be isolated.

A tentative formula for UDPG is presented in which uridine-5'-phosphate and glucose phosphate are joined, forming a pyrophosphate group.

The coenzymatic activity is destroyed by bromine, which affects the uracil moiety, and by alkali, which probably brings about a rearrangement of the molecule.

A micromethod for the measurement of the copper complex formation of nucleotides is presented.

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ON THE ORIGIN OF BILE PIGMENT IN NORMAL MAN*

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It has usually been assumed that bile pigment in the mammal is derived exclusively, or very nearly so, from the hemoglobin of mature, circulating red blood cells. Studies in the dog have revealed a close correspondence between the measurements of bile pigment excretion and the calculated degradation of hemoglobin of circulating red cells (3). A review of other studies in man, however, reveals less consistent results (4). The variations in results may be due merely to technical difficulties in the measurement of bile pigment or to errors in the assumptions that the hemoglobin of circulating erythrocytes is quantitatively converted to, and is the sole source of, bile pigment. In any case, conclusive proof has not been adduced for these assumptions.

Another view, proposed by Whipple (5), was that bile pigment is derived to a significant extent from sources other than the hemoglobin of circulating erythrocytes. Conclusive experimental evidence for the existence and identity of other sources has, however, not been available.

The studies described in this report offer evidence pertinent to the problem of the biologic origin of bile pigment. Previous studies have shown that glycine is specifically utilized in the biosynthesis of protoporphyrin (6) and that the administration of N^{15} -labeled glycine provides a method for the determination of the average life span and pattern of destruction of the human erythrocyte in normal and pathologic states (7, 8). It was found that in normal man the average life span of the erythrocyte is approximately 120 days. It appears that very few of the mature, circulating erythrocytes are destroyed before the 40th day. Accordingly, if bile pigment is derived solely from the hemoglobin of mature, circulating erythrocytes, no significant concentration of N^{15} should be found in the bile pigment during the first 6 weeks after the administration of N^{15} -labeled glycine. With the onset of destruction of red cells contain-

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ing labeled heme the isotope concentration in the bile pigment should reflect the destruction of the cells containing labeled hemoglobin.

Stercobilin was isolated from the feces and hemin from the circulating erythrocytes of two normal, male, adults following the administration of N^{15} -labeled glycine. The N^{15} concentrations in hemin and stercobilin were determined. The results of the experiment in which isotopic glycine

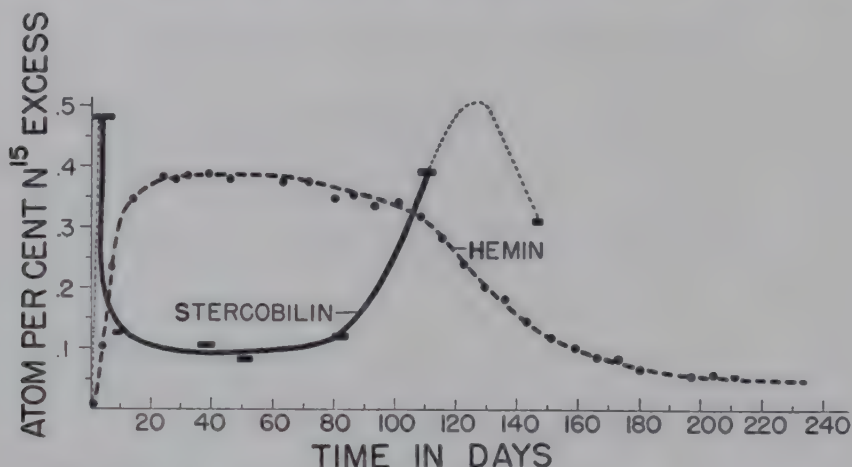


FIG. 1. N^{15} concentration in hemin and stercobilin of a normal man after the start of feeding N^{15} -labeled glycine for 2 days.

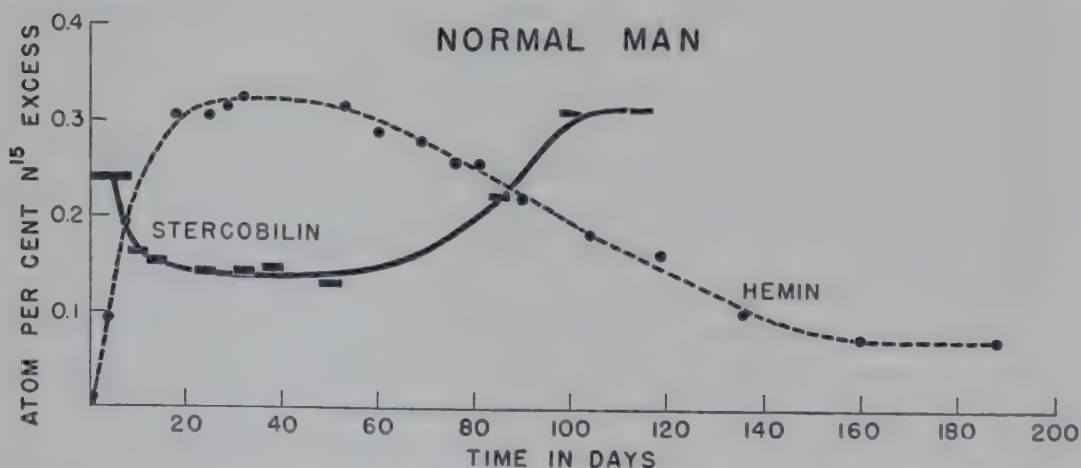


FIG. 2. N^{15} concentration in hemin and stercobilin after the start of intravenous administration of N^{15} -labeled glycine over a 2 day period.

was fed orally are presented in Fig. 1; the results in which glycine was administered intravenously are shown in Fig. 2.

The curve of N^{15} concentration in hemin of the first subject has been presented previously (8). The average life span of the erythrocytes in this subject is 120 days, and half (the second and third quarters) of the cell population is destroyed within a time span of 35 days (106 to 141 days).

There appears to be no significant destruction of mature, circulating erythrocytes prior to the 40th day. The N^{15} concentration in the stercobilin, isolated at intervals during the latter part of the experiment, reflects the destruction of cells containing labeled hemoglobin and the conversion of labeled heme to labeled bile pigment. From the 70th to the 190th day the major part of the decline in heme N^{15} occurs. During this period the N^{15} which disappears from the circulating heme approximates the amount of N^{15} which appears in the stercobilin. The isotope concentration of the 34 gm. of circulating heme (8) declined from 0.35 to 0.06 atom per cent excess; 9.6 mg. of N^{15} disappeared from the circulating heme. We estimate that during this period the average N^{15} concentration of the excreted stercobilin was approximately 0.24 atom per cent excess. With a normal excretion of 250 to 300 mg. of bilirubin per day the total amount of N^{15} excreted during this period would be 7.5 to 9 mg. of N^{15} . This represents reasonably good agreement and indicates that the major part of bile pigment is indeed derived from the hemoglobin of circulating red blood cells.

During the first 8 days of the experiment, however, at a time when there is apparently no destruction of mature, circulating erythrocytes containing labeled hemoglobin, the N^{15} concentration in the stercobilin is very high. This indicates that a portion of the stercobilin is not derived from the hemoglobin of mature, circulating erythrocytes. The magnitude of this portion may be estimated from the isotope concentration in the bile pigment and the average isotope concentration in newly formed heme during the 8 day period. It is reasonable to assume that the isotope concentration in newly formed heme, which presumably approximates that of its precursors, also approximates that of the precursors of this portion of bile pigment. The average N^{15} concentration in newly formed heme during the 8 day period may be determined by analysis of the curve of N^{15} concentration in hemin. Since the only circulating cells which contain labeled heme during this 8 day period have been formed during these 8 days, and since they represent only one-fifteenth ($8/120$) of the cells in circulation, the average isotope concentration in newly formed heme is actually 15 times the observed N^{15} concentration in the hemin of total circulating erythrocytes. The N^{15} concentration in the hemin of total circulating erythrocytes on the 8th day is 0.28 atom per cent excess N^{15} ; the average concentration in newly formed heme during this period is, accordingly, 4.20 atom per cent excess N^{15} . On the reasonable assumption that 4.20 atom per cent excess N^{15} approximates the isotope concentration in the precursors of stercobilin other than the hemoglobin of mature, circulating erythrocytes, the isotope concentration of 0.480 atom per cent excess N^{15} in the stercobilin indicates that *at least* 11 per cent ($0.480/4.20$)

of the stercobilin in this subject is derived from isotopically labeled precursors which are not the hemoglobin of circulating, mature red cells.

To obviate the possibility that the labeled stercobilin was derived from labeled glycine which had not been absorbed from the intestine and from which stercobilin was formed by intestinal bacteria, N¹⁵-labeled glycine was administered to a second normal subject by intravenous injection. The isotope concentrations in stercobilin and in hemin are shown in Fig. 2. The high isotope concentration in stercobilin during the first 8 days confirms the finding in the first subject and rules out the possibility that unabsorbed labeled glycine is the source of the labeled stercobilin.

The biologic source of stercobilin which is not derived from mature, circulating erythrocytes is not yet clearly defined. There are several possible sources.

Bile pigment may be formed from the hemoglobin of erythrocytes which are destroyed in the bone marrow before ever reaching the peripheral blood or from the hemoglobin of newly formed erythrocytes which are destroyed very shortly after entering the circulation. This possibility would suggest that the red cell population is composed normally not only of cells which reach maturity and survive for an average of 120 days, but also of cells which are destroyed in the bone marrow or within a very short time after their release into the circulation. The suggestion of a cell population with two component fractions which undergo destruction at markedly different rates is of interest in the light of findings in sickle-cell anemia which indicate the presence both of a rapidly destroyed component and of another which is more slowly destroyed (8). In any case, if a fraction of the cells in normal man is very rapidly destroyed, it is composed of cells which are young and which are to be differentiated from mature erythrocytes which survive, on the average, 120 days.

It is conceivable that some intracorpuseular degradation of hemoglobin and formation of bile pigment may occur in the developing erythrocytes in the bone marrow prior to their release into the peripheral blood. There is no evidence in support of this speculative possibility, however.

Other possible sources are myoglobin and catalase, peroxidase, and the cytochromes. It was found by Whipple and Róbscheit-Robbins that the injection of myoglobin into a dog resulted in increased urobilinuria (9). Although there is as yet no evidence that myoglobin in intact muscle cells is degraded and that its heme can then be converted to bile pigment, such a possibility must be considered. It is claimed that the heme of myoglobin constitutes 20 per cent of the total body heme (10). If one were to attribute the high isotope concentration in the stercobilin during the initial period of this experiment solely to the degradation of myoglobin, it would require that 5 per cent of the heme of myoglobin be regenerated

daily.¹ Such a rate of synthesis and degradation of the heme of myoglobin is consistent with the N^{15} values, but is not consistent with the known quantities of bile pigment excreted. The normal amounts of myoglobin heme and of hemoglobin heme in the adult human are approximately 8 and 32 gm. respectively (10). A rate of turnover of 5 per cent per day for myoglobin heme should result in the production of about 360 mg. daily of bile pigment; the known rate of degradation of heme (0.8 per cent per day) will result in the production of about 245 mg. daily of bile pigment. A total production of about 600 mg. per day for the normal adult human, however, is about twice as great as the reported values found experimentally (4). Furthermore these calculations require that 60 per cent of bile pigment be derived from myoglobin, a condition which is not consistent with the demonstration above that the major part of bile pigment is derived from hemoglobin.

If the rate of synthesis and degradation of the heme of myoglobin is less than 5 per cent per day, then the attainment of an isotope concentration of 0.48 atom per cent excess in the stercobilin would require that even more than 60 per cent of the bile pigment must be derived from myoglobin heme. If the rate of synthesis and degradation of the heme of myoglobin is greater than 5 per cent per day, then the total amount of bile pigment formed would be even greater than 600 mg. per day. These assumptions lead to untenable conclusions. Myoglobin therefore cannot be the sole source of the portion of bile pigment which is not derived from the hemoglobin of circulating erythrocytes. The possibility remains, however, that part of this portion of bile pigment may be derived from myoglobin and the respiratory heme pigments.

It is possible that heme or porphyrins which have not been utilized for hemoglobin production may be converted to bile pigment.

The observation that administration of N^{15} -labeled hematin to a dog results in a rapid formation of N^{15} -labeled stercobilin (11) indicates that

¹ Assume that the average isotope concentration in the newly formed heme of myoglobin is the same as that in the newly formed heme of hemoglobin. The latter value is 4.2 atom per cent excess for the first 8 day period. Further, let the fraction Y of the myoglobin be regenerated daily. Then to a first approximation the average isotope concentration in the total myoglobin heme during the first 8 days of the experiment will be $4.2(8Y)/2$. If the myoglobin heme accounts for 20 per cent of the total body heme and the heme of hemoglobin for essentially 80 per cent, then the fractional contribution of the heme of myoglobin to bile pigment will be $0.2Y$, while that of the heme of hemoglobin will be 0.8×0.008 . (Since the average erythrocyte life span is 120 days, 0.008 is the fraction of total hemoglobin degraded daily.) The isotope concentration of the excreted bile pigment will then be $0.2Y/[0.2Y + (0.8 \times 0.008)] \times 4.2/2 \times 8Y$. This by measurement we find to be 0.48 atom per cent excess. Solving for Y yields a value of 0.05.

heme which is not bound to globin may form bile pigment. If heme is formed in excess of that which is required, it is possible that the excess heme is converted to bile pigment.

The steps in the biologic synthesis of heme which mediate between glycine and acetic acid (12, 13) and the fully developed protoporphyrin molecule are as yet unknown. It is conceivable that other porphyrins are produced which are not (or are incompletely) utilized for hemoglobin production but might be converted to bile pigment, although the studies of Lemberg on the conversion of hemoglobin to bile pigment indicate that iron-free porphyrins are not normally intermediates in the conversion of heme to bile pigment (14).

Direct synthesis of bile pigment may occur via a pathway which does not involve degradation of a porphyrin ring. It is possible that a metabolic pool of pyrroles, derived from glycine and acetic acid, is utilized in the synthesis of porphyrins and of bile pigment.

It is noteworthy that in a subject with congenital porphyria, who excretes large quantities of uroporphyrin I and coproporphyrin I, the administration of N^{15} -labeled glycine for 3 days was attended by very high concentrations of N^{15} (2.5 atom per cent excess) in the stercobilin during the first 12 days in the absence of any apparent destruction of circulating erythrocytes containing labeled hemoglobin (15). The significance of this finding in terms of the possible derivation of bile pigment from iron-free porphyrins or pyrroles is discussed in an accompanying paper (15).

It should be noted that there is an appreciable isotope concentration in stercobilin from about the 12th to 80th day. The rise in isotope concentration which occurs after the 80th day is readily ascribable to the destruction of circulating erythrocytes containing labeled hemoglobin. The initial rise in N^{15} concentration during the first 8 days is due to the formation of bile pigment from one or more of the sources (which are discussed above) at a time when the N^{15} concentration in the body glycine is relatively high. The isotope concentration in the glycine falls, however, to very low levels within a few weeks after the cessation of feeding labeled glycine. Accordingly, the finding in stercobilin of N^{15} concentrations which are greater than that which obtains in body glycine during the same period of time indicates that at least part of the labeled stercobilin found at this time is derived from one or more sources which were themselves formed at an earlier time when the N^{15} concentration in glycine was high. The rate of turnover of these bile pigment precursors is slow compared to the rate of turnover of the precursors responsible for the high concentration of N^{15} in the 1st week of the experiment. Only when the rates of turnover of all the precursors of bile pigment are established will it be possible to evaluate quantitatively the contributions of each to the formation of bile pigment.

EXPERIMENTAL

Both subjects were white, male, medical students, the first 23 years old, the second 22 years of age. Each was in good health and had had no serious illness in the past. There was no history of blood dyscrasia in their families. Hematologic findings in both subjects were normal and remained essentially unchanged during the study.

Labeled glycine was prepared from potassium phthalimide and chloroacetic ester (16). The first subject ingested 48 gm. of glycine labeled with 31 atom per cent excess N^{15} over a 48 hour period. The glycine was fed hourly in equal doses except for triple doses at 12 midnight and 3 a.m., with no other doses between 12 midnight and 6 a.m. The second subject received labeled glycine intravenously over a 48 hour period. Glycine labeled with 32 atom per cent excess N^{15} was injected in the antecubital veins in 10 per cent aqueous solution. Injections of 20 ml. were given at the start of the experiment and at 5, 19, 24, 29, and 43 hours; 40 ml. injections were given at 10 and 34 hours.

Hemin was isolated by the usual procedure (17) from 20 to 30 ml. samples of venous blood drawn at frequent intervals during the course of the experiments.

Stercobilin was isolated by a procedure which is modified only slightly from an unpublished method of Watson.² Stools were collected in dark bottles for 4 or 8 day periods. The stools were repeatedly extracted with $CHCl_3$ and glacial acetic acid in a ratio of about 4:1 until the extract was only faintly colored. The combined extracts were concentrated *in vacuo* to a very small volume and then aerated overnight to complete the oxidation of stercobilinogen to stercobilin. The residue was then extracted repeatedly with hot 7 per cent HCl. The HCl solution was in turn extracted repeatedly with $CHCl_3$. The combined $CHCl_3$ extracts were concentrated *in vacuo* to a very small volume, at which point crystallization often occurred. Crystallization was accelerated by the addition of a small volume of dry acetone. Almost all of the samples were repeatedly recrystallized from $CHCl_3$; further recrystallization and determination of N^{15} concentration of many of the samples revealed essentially constant isotope contents.

Optical rotations of the stercobilin samples were performed on $CHCl_3$ solutions containing 20 to 30 mg. per 100 ml. The results were as follows: first subject, days 0 to 8, $[\alpha]_D^{20} = -3340^\circ$; days 36 to 40, $[\alpha]_D^{20} = -3550^\circ$; days 49 to 53, $[\alpha]_D^{20} = -3650^\circ$; days 80 to 84, $[\alpha]_D^{20} = -3780^\circ$; days 108 to 112, $[\alpha]_D^{20} = -3610^\circ$; second subject, days 0 to 8, $[\alpha]_D^{30} = -3480^\circ$; days 9 to 12, $[\alpha]_D^{30} = -3330^\circ$; days 15 to 18, $[\alpha]_D^{30} = -3440^\circ$; days 21 to 24, $[\alpha]_D^{31} = -3360^\circ$; days 29 to 32, $[\alpha]_D^{30} = -3380^\circ$; days 36 to 39, $[\alpha]_D^{30} =$

² Watson, C. J., personal communication.

-3550° ; days 51 to 54, $[\alpha]_D^{28} = -3660^\circ$; days 87 to 90, $[\alpha]_D^{28} = -3700^\circ$; days 103 to 106, $[\alpha]_D^{28} = -3670^\circ$.

SUMMARY

Although the major portion of bile pigment in normal man is derived from the hemoglobin of mature, circulating erythrocytes, a significant portion (at least 11 per cent) is derived from one or more additional sources. The possible nature of the additional sources is discussed.

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THE FORMATION OF BILE PIGMENT IN PERNICIOUS ANEMIA*

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Studies on the origin of bile pigment in normal man have revealed that a significant portion of bile pigment is derived from one or more sources other than the hemoglobin of mature, circulating erythrocytes (3). This finding suggested the possibility that the markedly increased quantities of bile pigment excreted in untreated pernicious anemia might also be derived in part from sources other than the hemoglobin of mature, circulating erythrocytes. Such a possibility is not in accord with the assumption, made by most investigators in the past, that the increased quantities of bile pigment which are excreted are the result solely of an abnormally increased rate of hemolysis (4). This assumption has been challenged by others (5, 6) who have held that the rate of turnover of hemoglobin and circulating erythrocytes which would be required to produce such large quantities of bile pigment was improbably high. Conclusive evidence in support of either position, however, was not available. The study described in this report was performed to provide information which might help to elucidate the relationship of hemoglobin metabolism to the formation of bile pigment in pernicious anemia.

The administration of glycine labeled with N^{15} affords a method for the study of the life span and pattern of destruction of the human erythrocyte in normal and pathologic states (7, 8). This method was used to study heme synthesis and dynamics of the red blood cell in a patient with the characteristic symptoms of pernicious anemia. The curve of N^{15} concentration in the hemin of this subject has been described previously (8). At the start of the experiment the patient had received no antianemia therapy. Glycine labeled with N^{15} was fed for 2 days. After 15 days, when the isotope concentration in the hemin appeared to be approaching its maximum value, it was considered inadvisable to withhold treatment longer, and liver extract in large dosage was administered. Analysis of the data re-

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vealed that the erythrocyte population was mixed, some of the cells enjoying a true life span and others being destroyed at random; the mean survival time of the erythrocytes was approximately 85 days. The rate of production of circulating red cell hemoglobin was about four-fifths the normal; the rate of production of circulating red cells about half the normal rate.

Stercobilin was isolated from 4 day collections of stools at intervals during the course of the same study and the N^{15} concentrations of the stercobilin samples determined. The isotope concentrations in the hemin and

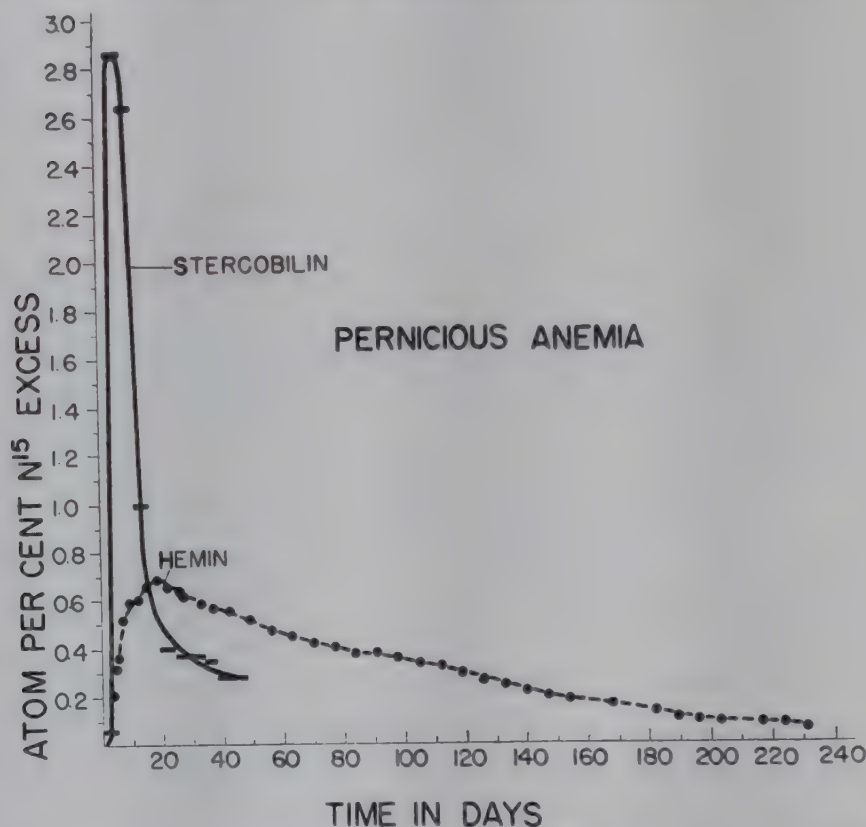


FIG. 1. N^{15} concentrations in hemin and stercobilin after the start of feeding N^{15} -labeled glycine for 2 days.

the stercobilin are shown in Fig. 1. The curve of N^{15} concentrations in stercobilin reveals strikingly high values from the 5th to the 12th day. The low value during the first 4 days is most likely a reflection of the lag in excretion of bile pigment after its formation, with isotopically labeled stercobilin diluted by unlabeled stercobilin formed prior to the administration of labeled glycine. The very high N^{15} concentrations attained between the 5th and 12th days indicate that a large fraction of the bile pigment is derived from a source other than the hemoglobin of mature, circulating erythrocytes. The magnitude of this fraction may be estimated from the

isotope concentration in the bile pigment and the average isotope concentration in newly formed heme during an equivalent period. From the 5th to 12th day, the N^{15} concentration in the stercobilin was approximately 2.75 atom per cent excess N^{15} . If one assumes a lag in this patient of about 3 days between the degradation of hemoglobin of circulating erythrocytes and the excretion of the resultant bile pigment in the feces, the equivalent 8 day period for the estimation of the average isotope concentration of newly formed heme is from the 2nd to the 9th day. The average isotope concentration in the newly formed heme during this period is approximately 6.3 atom per cent excess N^{15} (for this type of calculation see the previous paper (3)). During this period, a portion of the newly formed circulating cells containing labeled heme undergoes destruction. The average N^{15} concentration of the heme in the circulation between the 2nd and 9th days is 0.3 atom per cent excess N^{15} . If one assumes completely random destruction of the cells, the isotope concentration in bile pigment derived from the destruction of cells containing labeled heme during this period would have the same value. Inasmuch as a portion of the newly formed labeled cells may not be destroyed at random, the isotope concentration in the bile pigment derived from the destruction of circulating cells is probably somewhat less than 0.3 atom per cent excess N^{15} . Let us accept this as an upper limiting value, however.

The magnitude of the fraction of bile pigment which is not derived from the hemoglobin of circulating erythrocytes may be estimated as follows: If α is the fraction which is derived from the hemoglobin of circulating erythrocytes, then 0.3α is the contribution of heme of circulating erythrocytes to the isotope content of the bile pigment. The remainder of the bile pigment ($1 - \alpha$) is derived from sources which have, as an upper limit, an average isotope concentration similar to that of newly formed heme. The average isotope concentration of newly formed heme is approximately 6.3 atom per cent excess N^{15} . The contribution of these other sources to the isotope content of the bile pigment is $6.3(1 - \alpha)$. Then $0.3\alpha + 6.3(1 - \alpha) = 2.75$. Solving for α yields a value of 0.59; *i.e.*, at most about 60 per cent of the bile pigment is derived from the hemoglobin of circulating erythrocytes and at least about 40 per cent is derived from other sources.

These findings provide an explanation for the discrepancy in untreated pernicious anemia between the very high levels of bile pigment production and the relatively moderate rates of destruction of circulating red blood cells. The possible sources for that portion of bile pigment (at least 40 per cent) which is not derived from the hemoglobin of mature, circulating erythrocytes are similar to those which have been considered in normal man.

Bile pigment may be formed from the hemoglobin of erythrocytes which are destroyed in the bone marrow before they reach the peripheral blood or from the hemoglobin of newly formed erythrocytes which are destroyed shortly after entering the circulation. The studies of Peabody and Broun on the phagocytosis of erythrocytes in specimens of bone marrow obtained post mortem indicate that, whereas phagocytosis occurs to a very limited extent in normal marrow, it is quite marked in the active stages of pernicious anemia (9). This finding, however, was observed by Watson *et al.* (10) to be only an irregular occurrence. It seems reasonable that a portion of the abnormal cells in the bone marrow may be particularly vulnerable to destruction and may not even survive the transition to the circulating blood. In addition, of those cells which do reach the circulation a portion may be destroyed shortly after release from the bone marrow. Abnormal reticulocytes which survive apparently for a very short time in the peripheral blood have been observed by Heilmeyer and Eilers (11). The destruction of young cells may result from structural abnormality and the ordinary trauma to which erythrocytes are exposed normally, or it may result from unusual susceptibility of these abnormal cells to hemolytic agents which are thought by some workers (12-15) to be present in the blood of some patients with untreated pernicious anemia. In any case, destruction of very young cells is probably a more prominent factor in the production of bile pigment in this disease than in normal man.

The contribution of myoglobin and the respiratory heme pigments to bile pigment formation in this disease is unknown, as is the rate of turnover of myoglobin. It seems unlikely, in the light of the considerations presented in the previous paper (3), that myoglobin accounts for a large part of this portion of bile pigment.

Heme which has not been utilized for hemoglobin production may serve as a source of bile pigment in this disease. It is possible that inadequate amounts of globin are produced or that the binding of available heme and globin is incomplete. In either case, the excess or unbound heme could be converted to bile pigment. Hematinemia has been reported to occur in pernicious anemia (16). That hematin which is not bound to globin can be converted to bile pigment is indicated by the finding that the intravenous administration of isotopic hematin results in the formation of isotopic stercobilin (17).

Pernicious anemia is characterized by the excretion of increased amounts of coproporphyrin I; following treatment with liver extract, the excretion declines to normal levels (18). Increased excretion of coproporphyrin I, which may be a by-product in the formation of porphyrins related to the etioporphyrin III configuration, may reflect increased formation of porphyrins of the III type. If there is a partial block in the conversion of

iron-free porphyrin to heme, the accumulated porphyrin may be directly converted to bile pigment. With the administration of antianemia therapy, the block in the conversion of porphyrin to heme would disappear and a simultaneous decline in bile pigment formation and rise in heme production would occur.

Finally, it remains to be determined whether bile pigment can be formed from pyrroles or pyrrole precursors directly, without prior conversion to a porphyrin ring.

As yet it is not possible to determine the sources which are responsible for the production of the large fraction of the bile pigment which is not derived from the hemoglobin of circulating erythrocytes. However, destruction of defective cells in the bone marrow probably plays a more important rôle in the formation of bile pigment in pernicious anemia than it does in normal man.

EXPERIMENTAL

The subject was a 51 year-old man whose history, physical findings, and hematologic data were characteristic of untreated pernicious anemia. The data have been presented in detail previously (8).

The subject received 48 gm. of glycine containing 31.7 atom per cent excess N^{15} over the course of 48 hours. The glycine was fed hourly in equal doses, except for triple doses at 12 midnight and 3 a.m., with no other doses between 12 midnight and 6 a.m.

Hemin and stercobilin were isolated by the methods described in the previous paper (3). Optical rotations of some of the stercobilin samples were performed on $CHCl_3$ solutions containing 20 to 30 mg. per 100 ml. The results were as follows: days 0 to 4, $[\alpha]_D^{20} = -3040^\circ$; days 5 to 8, $[\alpha]_D^{30} = -3500^\circ$; days 9 to 12, $[\alpha]_D^{30} = -3600^\circ$; days 21 to 24, $[\alpha]_D^{30} = -3580^\circ$; days 25 to 34, $[\alpha]_D^{30} = -3620^\circ$; days 35-38, $[\alpha]_D^{30} = -3560^\circ$.

SUMMARY

In untreated pernicious anemia a large part of bile pigment is derived from one or more sources other than the hemoglobin of mature, circulating erythrocytes.

We wish to acknowledge the valuable assistance of Miss Martha Yamasaki and Mr. Alexander Korzun and to thank Mr. I. Sucher for the isotope analyses.

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PORPHYRIN FORMATION AND HEMOGLOBIN METABOLISM IN CONGENITAL PORPHYRIA*

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The studies reported here were conducted on a patient with congenital porphyria who excretes large amounts of uroporphyrin I and coproporphyrin I. They were directed to the following problems: (a) to determine whether glycine is specifically utilized in the biologic synthesis of porphyrins related to the etioporphyrin I configuration as it is utilized in the synthesis of protoporphyrin which is related to the etioporphyrin III configuration (1); (b) to measure the rates of formation (and degradation) of uroporphyrin I and coproporphyrin I; (c) to investigate the life span and pattern of destruction of the erythrocyte and the origins of bile pigment in this disease.

Material and Methods

The subject was a 15 year-old white girl¹ previously reported by Dobriner, Strain, and their associates (2, 3). She has enjoyed good health except for the manifestations of congenital porphyria, namely photosensitivity, brownish discoloration of the teeth, and excretion of large amounts of uroporphyrin I and coproporphyrin I. Physical examination in March, 1949, revealed no other abnormal findings.² Laboratory data at that time included the following: red blood cells, 4.54 million per c.mm. of blood; hemoglobin, 12.5 gm. (99 per cent) per 100 ml. of blood; hematocrit, 41.0 per cent; reticulocytes, 5.9 per cent; white blood cells, 5800 per c.mm. of blood; and a normal differential white cell count. Examina-

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† Died May 20, 1949.

¹ We are indebted to Dr. W. H. Strain, Strong Memorial Hospital, Rochester, New York, who afforded us the opportunity of studying this patient; his continued interest and help were invaluable in the performance of the study.

² We are grateful to Dr. R. W. Davis, Dr. R. M. Christian, and Dr. L. E. Young for permission to report these results of their physical and laboratory examinations, which were performed at the Strong Memorial Hospital, Rochester, New York.

tion of sternal bone marrow in January, 1949, revealed erythroid hyperplasia. A Coombs test for the presence of a circulating autoagglutinin was negative.

Glycine labeled with 32 atom per cent excess N^{15} (4) was given in doses of 4.0 gm. every 3 hours during the day for a total of five doses per day; 60 gm. of glycine were administered over the course of 3 days.

Uroporphyrin I was isolated from 4 day samples of urine and coproporphyrin I was isolated from 4 day stool collections. The isolation procedures were those described by Watson (5) and by Grinstein, Schwartz, and Watson (6). The methyl esters of uroporphyrin I and coproporphyrin I were prepared. The melting points of the various samples of uroporphyrin I methyl ester and of coproporphyrin I methyl ester are shown in Table I.

TABLE I
Melting Points of Methyl Esters of Uroporphyrin I and Coproporphyrin I

Days	Uroporphyrin I methyl ester	Coproporphyrin I methyl ester
	°C.	°C.
0- 4	284-287	245-253
5- 8	291-294	247-253
9-12		251-255
13-16	284-288	247-253
21-24		245-254
25-28	290-293	247-253
29-32		249-253
33-36	283-288	245-251
50-53		247-254
65-68	283-288	246-254
81-84	283-291	

Stercobilin was isolated from the stools according to a method of Watson (7). Hemin was prepared from 20 to 30 ml. of venous blood by the usual procedure (8). N^{15} analyses were carried out in the mass spectrometer.

RESULTS AND DISCUSSION

The N^{15} concentrations in the uroporphyrin I and in the coproporphyrin I following the start of labeled glycine feeding are plotted in Fig. 1. The maximum isotope concentration in the uroporphyrin I (4.65 atom per cent excess N^{15}) is reached during the first 4 days, after which there is a rather rapid decline. The maximum isotope concentration in the coproporphyrin I (6.82 atom per cent excess N^{15}) is reached during the second 4 day collection period. This lag in attaining maximum concen-

tration in the coproporphyrin is most likely a reflection of delayed excretion by the intestine. These high N^{15} values in uroporphyrin I and coproporphyrin I are of the same order of magnitude as the N^{15} concentration in the newly formed heme. The isotope concentration in newly formed heme during the first 8 days may be determined by analysis of the curve of N^{15} concentration in hemin (Fig. 2). The N^{15} concentration in the total circulating heme on the 8th day is 0.53 atom per cent excess

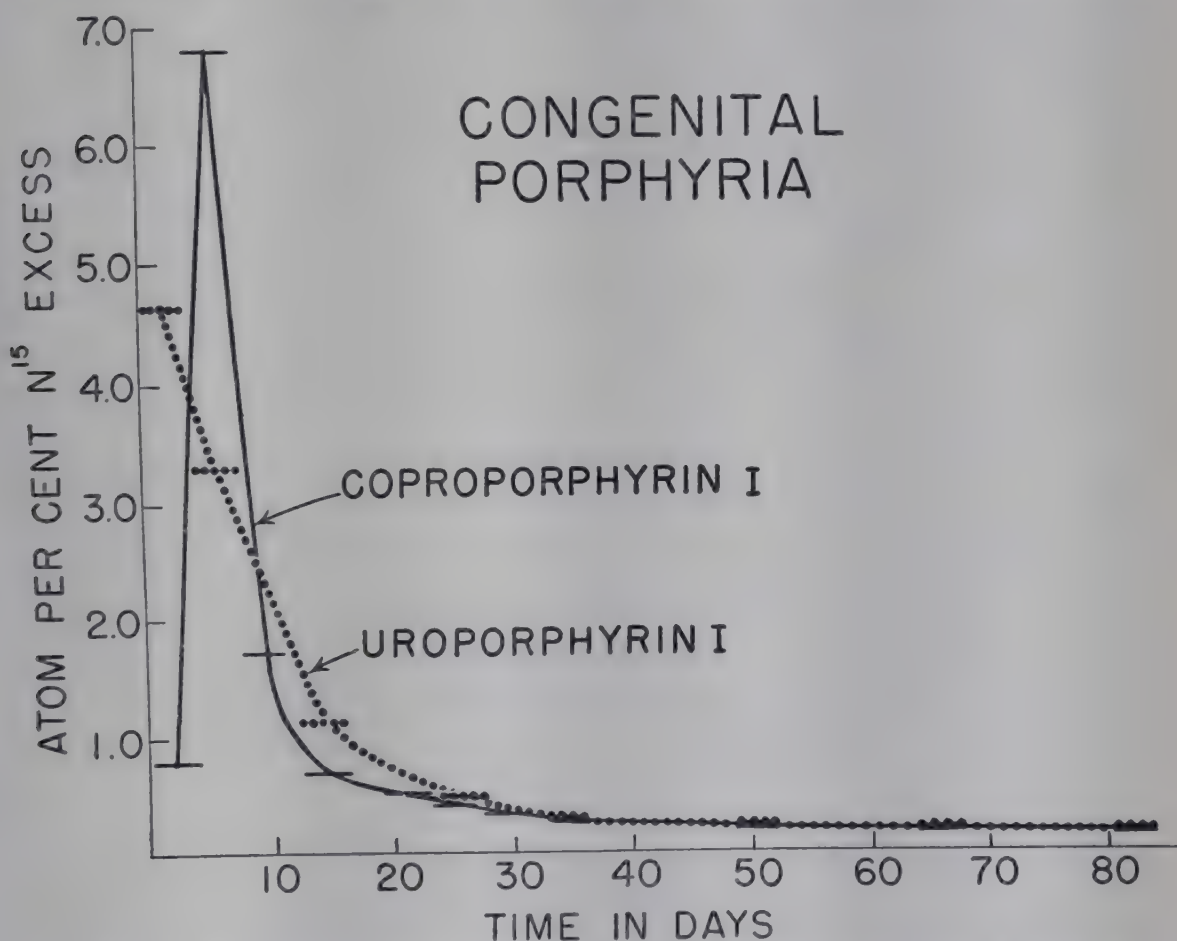


FIG. 1. N^{15} concentrations in uroporphyrin I and coproporphyrin I during and after the feeding of N^{15} -labeled glycine for 3 days.

N^{15} . The average isotope concentration in the newly formed heme during this 8 day period is equal to $(0.53 \times \text{average erythrocyte life span (days)})/8$ days since the only circulating cells which contain labeled heme have been formed during these 8 days. The average life span of the erythrocytes in this subject is approximately 120 days. The average isotope concentration in the newly formed heme is accordingly about 8.0 atom per cent excess N^{15} . That lower isotope concentrations are reached in the uroporphyrin I and coproporphyrin I may be due to the

presence of pools of these substances with dilution of isotopically labeled molecules by non-isotopic molecules formed prior to the ingestion of labeled glycine. In the case of heme, the dilution of newly formed isotopic heme by previously formed non-isotopic heme can be calculated, since the total circulating heme can be measured. The dilution of the isotope concentrations in the newly formed uroporphyrin I and coproporphyrin I by previously formed non-isotopic molecules cannot be determined precisely, because the size of the pools of these substances is not known. Since glycine is specifically utilized in the biologic synthesis of protoporphyrin (1) and the maximum isotope concentrations in the uroporphyrin I and coproporphyrin I are of a magnitude similar to that in newly formed heme, it can be concluded that glycine is specifically utilized in the biologic synthesis of porphyrins related to the etioporphyrin I configuration.

The decline in the isotope concentrations in the uroporphyrin I and coproporphyrin I provides some indication of the rates at which these porphyrins are synthesized. From the declining curves one can estimate t_1 , the half life time of these substances in the organism, *i.e.* the time in which half the molecules which are formed at one time have disappeared from the organism. It should be emphasized that these values for t_1 are maximum. As measured from the declining portion of each curve, the t_1 of coproporphyrin I is about 3 to 4 days, of uroporphyrin I about 6 to 7 days. These values represent rapid rates of turnover, and, since these values are maximum, the actual rates of synthesis of these porphyrins may be even more rapid. It can be concluded that with turnover times ($t_1 \times (1/\ln 2)$) of 5 and 9 days for coproporphyrin I and uroporphyrin I, respectively, the rates of synthesis (and degradation) for these substances are at least 20 and 11 per cent per day of their pools.

It is tempting to speculate on the relationship between uroporphyrin I and coproporphyrin I on the basis of their isotope concentrations. It is not as yet known whether coproporphyrin may be derived from uroporphyrin by decarboxylation of the acetic acid substituents, or whether the reverse reaction may occur by carboxylation of the methyl substituents of coproporphyrin. It has been suggested by Grinstein *et al.* (9), on the basis of findings in another case of congenital porphyria, that uroporphyrin may be derived from coproporphyrin. Their study, utilizing the same techniques, revealed a very high N^{15} concentration in coproporphyrin I initially and its immediate rapid decrease accompanied by an increase of the N^{15} concentration of the uroporphyrin I. The requirement that the maximum concentration in the product should never be greater than the maximum concentration of the precursor is a necessary but not sufficient condition in a homogeneous system only. In a heterogeneous system, the data are susceptible to an alternative explanation. If copro-

porphyrin were derived from uroporphyrin before either porphyrin enters its metabolic pool, the relative isotope concentrations in the excreted porphyrins would depend on the amounts of these porphyrins made per day and on the sizes of their metabolic pools. If the dilution of newly formed coproporphyrin were less than that of newly formed uroporphyrin, the isotope concentration in the coproporphyrin would be greater than that in the uroporphyrin even though the coproporphyrin were derived from uroporphyrin. Until further information is available on the routes of biologic synthesis of porphyrin compounds, definitive conclusions concerning

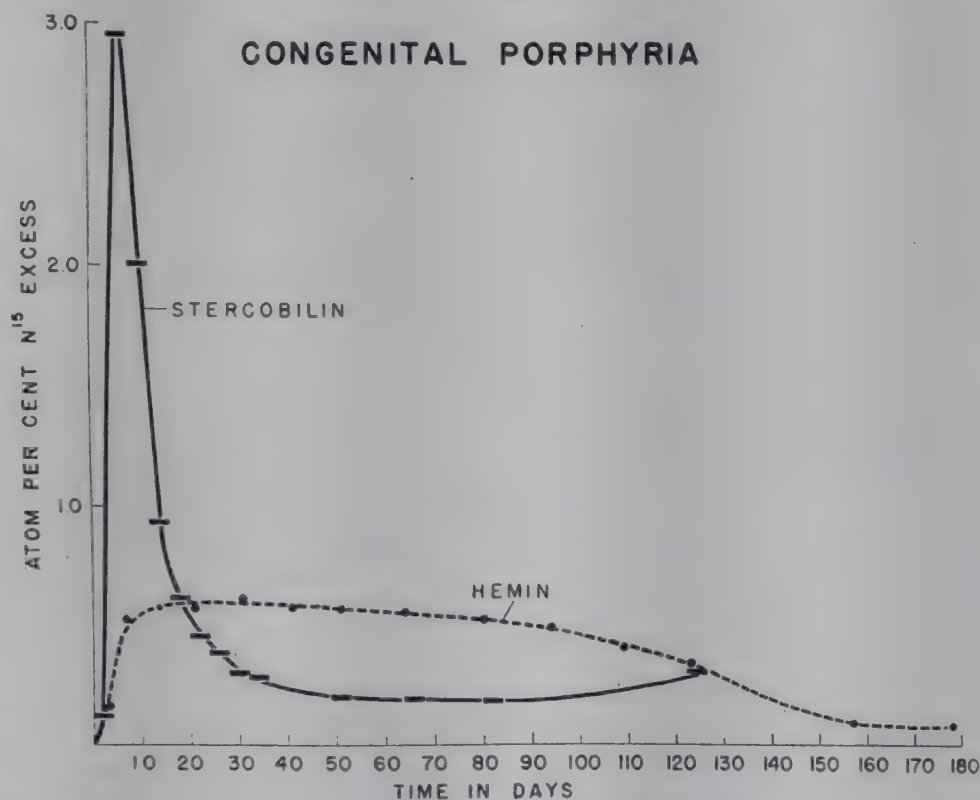


FIG. 2. N^{15} concentrations in hemin and stercobilin following the start of feeding labeled glycine for 3 days.

the interrelations of uroporphyrin I and coproporphyrin I cannot be derived from such data.

Fig. 2 shows the N^{15} concentrations in hemin and stercobilin. The curve of N^{15} concentration in hemin reveals a normal pattern of red cell survival and a normal average erythrocyte life span of about 120 days. These findings are of interest in the light of the reticulocytosis (about 6 per cent) in the peripheral blood and the erythroid hyperplasia of the bone marrow which are generally considered to be associated with increased erythropoiesis. In the absence of anemia or polycythemia and in the presence of a normal pattern of erythrocyte survival and a normal aver-

age erythrocyte life span, there is no other evidence of increased erythropoiesis. The reticulocytosis may reflect an earlier release of reticulocytes from the bone marrow or a longer persistence of erythrocytes in their reticulated form than is usual.

The curve of N^{15} concentrations in the stercobilin reveals strikingly high values during the first 2 weeks of the experiment. Since there is no apparent destruction of labeled, mature, circulating erythrocytes at this time, the high isotope concentrations in the bile pigment indicate that a large portion of the bile pigment in this disease is derived from one or more sources other than the hemoglobin of mature, circulating erythrocytes. This finding is in confirmation of our earlier findings in normal man and in pernicious anemia (10, 11). By calculations similar to those employed in the previous publications (10, 11), it can be shown that at least 31 per cent of the stercobilin of this patient is derived from sources other than the hemoglobin of mature, circulating erythrocytes. (N^{15} concentration in stercobilin (2.5))/(N^{15} concentration in newly formed heme (8.0)) = 0.31.

Despite the normal pattern of red cell destruction and the normal average erythrocyte life span, it is possible that a component fraction of the erythrocyte population is destroyed while still in the bone marrow or shortly after entering the circulation. Such destruction of young erythrocytes might be reflected in increased erythropoietic activity, and the hemoglobin of these destroyed cells could be rapidly converted to bile pigment.

The production of large amounts of porphyrin may require an active synthesis of large quantities of pyrroles. If bile pigment can be formed directly from pyrroles, the high N^{15} concentration in the stercobilin which is observed early in the experiment may be due to the formation of stercobilin from an active pool of pyrroles which may exist in this disease.

An additional possibility may be considered; namely, that uroporphyrin III and coproporphyrin III may also be made in increased quantities but that they are very rapidly degraded to bile pigment and hence do not accumulate and are not detected.³

These possibilities are stressed, although the possible contributions of other sources which have been considered previously (10, 11) should not be overlooked. The other possible sources are myoglobin and the respiratory heme pigments, and heme which is not utilized for hemoglobin production.

³ It is conceivable that stercobilin may be derived from porphyrins of the etioporphyrin I isomer configuration as well as from porphyrins of the etioporphyrin III structure. If this is true (and, it should be stated, there is no evidence to support this speculative possibility), the labeled stercobilin in this subject might be derived in part from the isotopically labeled uroporphyrin I and coproporphyrin I.

SUMMARY

1. Glycine is specifically utilized in the biologic synthesis of porphyrins related to the etioporphyrin I configuration.
2. The rates of formation and degradation of uroporphyrin I and of coproporphyrin I in a subject with congenital porphyria are found to be rapid.
3. A normal average erythrocyte life span of about 120 days was observed in this subject with congenital porphyria.
4. A large part, at least 31 per cent, of the stercobilin in this subject is derived from sources other than the hemoglobin of mature, circulating erythrocytes. The nature of these possible sources is discussed.

We wish to acknowledge the valuable assistance of Miss Martha Yamasaki, Miss Gloria Sabella, and Mr. Alexander Korzun and to thank Mr. I. Sucher for the isotope analyses.

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THE CONVERSION OF HEMATIN TO BILE PIGMENT*

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The rôle of hematin in hemoglobin and bile pigment metabolism is not clearly defined. Opinion has been divided over the questions of whether or not hematin is a normal degradation product of hemoglobin and whether or not it is converted to bile pigment. Bingold observed that the occurrence of hematin in the plasma is associated with a variety of pathologic conditions (1). The conditions in which hematinemia has been observed by him and others include hemolytic anemias, hemoglobinuria, pernicious anemia, severe liver disease, and large blood extravasations. It has been noted in the fetus during the second half of gestation and in umbilical cord blood (2). Duesberg (3) claimed that under normal conditions hemoglobin is quantitatively converted to bile pigment and that hematin appears only in pathologic conditions. The studies of Fairley (4) indicate that hematin in the plasma unites immediately with albumin to form methemalbumin. This substance is not found normally; it occurs, according to Fairley, as a result of intravascular hemolysis, whereas normally hemoglobin degradation occurs in the reticulo-endothelial system and is converted to bile pigment without an intermediate stage of methemalbumin.

Attempts to determine whether hematin is converted to bile pigment have consisted for the most part in injections of hematin and measurements of bile pigment excretion. Brugsch and Kawashima (5) and Bénard *et al.* (6) found increased excretion of bilirubin in dogs, and Pass, Schwartz, and Watson (7) found increased excretion of urobilinogen in man following intravenous injection of hematin. On the other hand, no increase in bilirubin excretion in man was found by Duesberg (3), and Gitter and Heilmeyer (8) observed an increase in urobilinogen excretion in but one of two men following injection of hematin intravenously. The conclusion, on the basis of this type of experiment, that bile pigment has been derived from hematin is subject to the criticism that the increased bile pigment excretion may not represent actual conversion of hematin to bile pigment but may be an indirect effect of the injection of hematin. Studies of

* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

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Lemberg (2) revealed that the injection of mesohematin in rabbits is followed by an increased excretion of bile pigment which, however, is not derived from mesohematin. Furthermore, early histologic studies by Brown (9) and more recently by Anderson *et al.* (10) have been interpreted as indicating that hematin is very slowly metabolized; it would consequently be difficult to ascribe the rapid increases in bile pigment excretion which follow injection of hematin to actual conversion of hematin to bile pigment.

This report is concerned with a study which attempts to provide direct evidence relating to the problem of the conversion of hematin to bile pigment.

Material and Methods

A normal dog, weighing approximately 17 kilos, was injected intravenously with 800 mg. of N^{15} -labeled hematin in divided doses on 3 consecutive days and stools were collected for 9 days. Stercobilin was isolated from the stools and its N^{15} concentration was analyzed.

The labeled hematin was prepared as follows: 1.0 gm. of glycine labeled with 32 atom per cent excess N^{15} was injected into a duck subcutaneously each day for 10 days. The duck was exsanguinated on the 14th day and hemin was prepared from the erythrocytes by the usual procedure (11) and recrystallized (12). The isotope concentration in the hemin was 1.425 atom per cent excess N^{15} . On the 1st day, 200 mg. of hemin were dissolved in 2 ml. of 5 per cent Na_2CO_3 ; the solution was diluted with 25 ml. of distilled water and added to about 95 ml. of 0.9 per cent sodium chloride solution to make a total volume of 120 ml. The dog was anesthetized with nembutal given intravenously in a dose of 0.065 gm. per kilo. The hematin solution was injected by intravenous drip over the course of an hour.

A similar procedure was followed on the 2 successive days. On the 2nd and 3rd days 300 mg. of hemin were dissolved in 3 ml. of 5 per cent Na_2CO_3 ; 27 ml. of distilled water and 120 ml. of 0.9 per cent saline were added for a total volume of 150 ml. The anesthesia, which lasted about 5 hours, and the infusions were well tolerated. The dog ate well and suffered no apparent toxic reactions.

Stercobilin was isolated in crystalline form by a method of Watson (13). 36 mg. were obtained from the 9 day stool specimen. The optical rotation, performed on a $CHCl_3$ solution containing 25 mg. per 100 ml., was $[\alpha]_D^{20} = -3550^\circ$.

RESULTS AND DISCUSSION

The isotope concentration in the stercobilin was found to be 0.325 atom per cent excess N^{15} . This represents conversion of hematin to bile pig-

ment. The extent to which the injected hematin was converted to bile pigment can be estimated approximately. In a normal dog of 17 kilos, the total circulating hemoglobin should be about 180 gm., and the total circulating heme about 7 gm. With a normal average erythrocyte life span of about 120 days (14), the daily turnover of circulating hemoglobin is about 0.8 per cent per day. The daily synthesis and degradation of circulating heme should therefore be about 56 mg. ($7.0 \text{ gm.} \times 0.008$). In 9 days a total of about 500 mg. of circulating heme should be degraded on the assumption of quantitative conversion of heme to bile pigment. The amount of labeled hematin which has been converted to bile pigment may be estimated as follows: if α represents this amount, then 1.425 (atom per cent excess N^{15}) $(\alpha(\text{mg.})/(\alpha + 500 \text{ mg.}))$ is equal to 0.325 atom per cent excess N^{15} . Solving for α yields a value of 148 mg. Of the total amount of labeled hematin which has been injected, 18 per cent ($148/800$) has been converted to bile pigment during the 9 day period. It is quite possible that the ultimate extent of conversion is much greater, for further degradation of hematin to bile pigment may occur after the first 9 days.

The objection might be raised that the conversion of hematin to stercobilin occurs through the utilization of the hematin for hemoglobin formation and subsequent degradation of the hemoglobin to bile pigment. Such a mechanism, however, would involve the dilution of isotopic hemoglobin by large amounts of non-isotopic hemoglobin. Bile pigment derived from isotopic hematin by such a route would have an isotope concentration far lower than that which is found. The rate of conversion of hematin to bile pigment indicates that at least a portion of hematin is metabolized much more rapidly than was suggested by earlier studies (9, 10). The demonstration of conversion of hematin to bile pigment lends support to the view that that portion of bile pigment which is not derived from the hemoglobin of mature circulating erythrocytes may originate, at least in part, from heme or hematin which is not utilized for hemoglobin formation (15, 16).

SUMMARY

Hematin can be readily converted to bile pigment in the dog.

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EGG WHITE PROTEINS

I. ELECTROPHORETIC STUDIES ON WHOLE WHITE*

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Preliminary to studies on the ethanol fractionation of the proteins of chicken egg white, it appeared desirable to investigate the electrophoretic composition in further detail. Electrophoretic analyses of these proteins have been reported by several workers, in particular Longsworth, Cannan, and MacInnes (1) and Bain and Deutsch (2).

In previous electrophoretic analyses of egg white, it has not been found possible to retain the ovomucin component in solution because of its inherent solubility properties. Particular attention has been paid to this point, and the present paper reports conditions for the electrophoretic analysis of egg white containing substantially all of this component. Results on thirty-one runs, under these conditions, are used to derive mean values for composition and mobilities of the various components together with coefficients of variation. Results on six genetic lines of chickens indicate small but statistically significant differences in egg white composition.

EXPERIMENTAL

Materials—Eggs of known history were secured from the Iowa State College Poultry Farm within 4 hours of being laid. The whites were separated as completely as possible from the yolks, the chalazae removed with tweezers, and the whites blended in a Waring blender equipped with a rheostat to control speed. For electrophoretic analysis, egg white was diluted approximately 10-fold with the buffer used. Approximately 50 ml. of this solution were dialyzed with mechanical agitation for 24 to 48 hours at 2° against 2 liters of buffer. Final protein concentrations were adjusted to a constant refractive increment (ΔN) of 0.002 with an Abbe refractometer. This increment corresponds to a protein concentration of approximately 1.0 per cent.

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Taken from a thesis submitted by Richard H. Forsythe to the Graduate Faculty, Iowa State College, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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Electrophoresis—Electrophoretic analyses were carried out in the double length single section cell described by Longworth *et al.* (1). A modified Philpot-Svensson optical system was employed and a 35 mm. camera with a focal plane shutter was substituted for the usual plate holder. In standard runs, photographs were taken with a rectangular diagonal slit. All pattern measurements have been made on tracings enlarged approximately three times over the dimensions of the cell. Electrophoresis was usually continued until maximum resolution was obtained (15,000 to 18,000 seconds) under a potential gradient of 4.0 to 4.5 volts cm^{-1} . Where resolution of protein components was not complete, arbitrary separation of the areas was accomplished by dropping a vertical line from the minima between the peaks.

RESULTS AND DISCUSSION

Standard Electrophoretic Conditions and Identification of Components—Longworth *et al.* (1) recommended a pH of approximately 3.9 for the analysis of egg white. However, since ovomucoid, the albumins, and one globulin component are not well resolved, either from themselves or from the standing anomalies, at this pH they considered it necessary to use other conditions for a complete analysis. Bain and Deutsch (2), on the other hand, used a pH of 8.6; under these conditions the globulins and ovomucoid were not well resolved. One of the problems in the electrophoresis of egg white lies in the fact that at least one component, lysozyme, is isoelectric at a pH above 10, so that between pH 4.0 and 10.0 there are present both cationic and anionic species with a consequent possibility of interaction. This appears to be the most important consideration behind Longworth's recommendation of a low pH.

Electrophoretic runs were carried out over a pH range of 3.9 to 8.4. It was found that even at pH 3.9 (in acetate buffer) there was at least one anionic component. Hence, to eliminate possibilities of coulombic interaction, it would be necessary to go to an even lower pH, a situation which would obviously be dangerous from the standpoint of protein stability. Furthermore, it was found that blended or homogenized egg white could be diluted 10-fold with 0.15 M (or over) NaCl and dialyzed against a phosphate buffer of pH above 6.5 with no precipitation. This observation opened up the possibility of studying whole white (including ovomucin).

Comparative runs at pH 6.6 and 7.8 (phosphate) and pH 8.4 (veronal) indicated better resolution to be attained at 7.8. Furthermore, this pH was favored from the point of view of its being nearly that of normal fresh egg white. The effect of ionic strength at this pH was explored (Fig. 1) by varying the NaCl concentration from 0 to 0.15 M, while holding the phosphate constant at 0.05 ionic strength. The results are in qualitative agreement with theory, the mobilities increasing with decreasing ionic

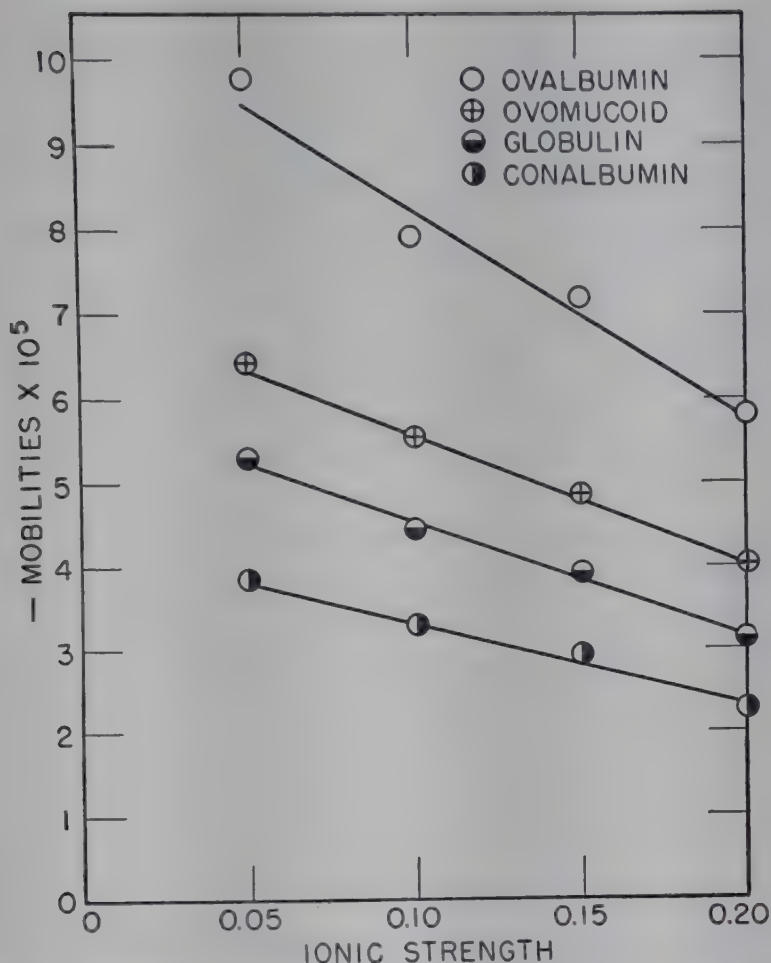


FIG. 1. Effect of ionic strength on the electrophoretic mobilities of the egg white proteins at pH 7.8. Average of ascending and descending mobilities from two runs at each ionic strength.



FIG. 2. Normal electrophoretic pattern of whole egg white at pH 7.8 (standard conditions).

strength. No improvement in resolution at low ionic strength was apparent. On the basis of these experiments, a buffer of pH 7.8 and ionic strength 0.05 in phosphate and 0.15 M in chloride was adopted for all routine runs.

A pattern obtained under the standard conditions is reproduced in Fig. 2. Resolution on the descending side leaves something to be desired, but is at least as good as that obtained under the other conditions which have

been used. Resolution on the ascending side is quite good if electrophoresis is prolonged to about 18,000 seconds. The lack of enantiography is characteristic also of earlier published work on egg white, and may be a result of interaction effects. The sharpening of the conalbumin boundary on the descending side is characteristic and may also be due to interactions. To check further on interactions, a number of experiments have been carried out on known mixtures of the various proteins under the same conditions. Mixtures studied include albumin lysozyme (at two ratios, 1:1 and in a ratio roughly the same as that in which they are present in whole white), ovomucoid lysozyme, and conalbumin lysozyme. All patterns were entirely normal with the exception of the albumin lysozyme mixture at a ratio of 1:1, in which case there was a sharpening of the albumin boundary on both sides and of the falling lysozyme boundary.

A word of explanation is required with regard to the labeling of the components in Fig. 2. That component migrating positively (on the descending side it has migrated off the pattern; it normally could be seen on both sides early in the course of the run) is labeled L (lysozyme) and has been so identified by isolating the solution from the top region of the descending limb and demonstrating the expected lytic activity by the method of Boasson (3). (An appropriate control was run on the solution from the other limb.) The component labeled X may be the third albumin component (A_3) reported by Cann (4). The next component is labeled O, ovomucoid, on the strength of isolation studies and studies on whole white enriched with this component. The next two peaks are labeled G_2 and G_3 , since it seems probable that these correspond to the components so named by Longsworth *et al.* (1).

Attempts were made to identify the ovomucin component as follows. Electrophoretic runs were made on normal white, white from which the mucin had been removed by centrifugation,¹ and white enriched approximately 25-fold with mucin. The results (Table I) are somewhat surprising in that no mucin peak can be identified, nor is there any appreciable alteration in the relative amounts of the other peaks. Unfortunately these runs were not prolonged to optimal resolution; reinvestigation of this problem is planned.

Normal Composition of White—In Table II are reported the results of thirty-one runs on pooled lots of egg white (each representing at least a dozen eggs). In about half of these, resolution was somewhat inferior to that shown in Fig. 2 because of discontinuance of electrophoresis at about 15,000 seconds. In particular component X was not resolved in all of these runs. Hence in this compilation the peak designated component X,

¹ This preparation technique is discussed in Paper II of this series.

where resolvable, was included with ovomucoid (since it would be so included in those instances in which it was not resolved). Also, no attempt is made in Table II to resolve components G_2 and G_3 . The variations indicated here are in part experimental but include also differences due to genetic strains, seasonal influences, and nutritional factors.

TABLE I

Comparison of Electrophoretic Compositions of Natural, Mucin-Free, and Mucin-Enriched Egg White

pH 7.8, $\frac{r}{2} = 0.2$, protein concentration 1.0 per cent. Averages of ascending and descending patterns.

	Natural	Mucin-free	Mucin-enriched
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Albumin.....	66.6	66.2	64.5
Ovomucoid.....	9.5	10.3	10.2
Globulin.....	9.0	8.1	9.5
Conalbumin.....	14.2	15.3	15.9

TABLE II

Relative Composition and Mobilities of Protein Constituents of Egg White under Standard Electrophoretic Conditions

Results of thirty-one runs.

	Mobility $\times 10^{-5}$ cm. ² volt ⁻¹ sec. ⁻¹				Per cent composition			
	Ascending		Descending		Ascending		Descending	
Ovalbumin.....	-5.88	8.8*	-5.78	8.5*	64.7	5.6*	65.1	4.5*
Ovomucoid.....	-4.16	13.2	-3.85	10.4	8.5	24.7	9.9	16.2
Globulin.....	-3.30	12.7	-3.03	12.2	8.5	21.1	8.9	19.1
Conalbumin.....	-2.41	13.7	-2.17	15.2	13.9	12.9	13.7	21.1
Lysozyme.....	+1.22		+1.10		4.5		2.3	

* Precision is expressed as the coefficient of variation.

The coefficients of variation in Table III indicate better the experimental reproducibility attainable under the conditions used. These coefficients of variation are somewhat higher than those attainable in the plasma protein system under the most favorable conditions, but are thought to be at least as good and probably better than those which have been attained in egg white under the conditions previously used.

TABLE III

Mean Electrophoretic Composition from Replicate Analyses of Egg White from Six Breed Lots of Chickens, Coefficients of Variation for Replicates, and Analyses of Variance Tests for Significance

F is the ratio of the variance between groups to that within groups.

Component		Mean composition*							<i>F</i>
		Group 1	Group 5	Group 16A	Group 16B	Group BAI-A	Group BAI-B	Mean Coefficient of variation	
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	
Conalbumin	Ascending	12.1	13.4	14.3	12.0	15.2	12.6	8.6	2.92
	Descending	10.2	12.4	13.1	13.4	15.7	15.0	7.1	5.80†
	Average	11.1	12.9	13.7	12.7	15.4	13.8	6.5	1.44
Globulin G ₂	Ascending	6.9		3.9	9.3			4.7	139†
	Descending	9.1		8.8	9.1			9.2	0.10
	Average	8.0		6.4	9.2			6.7	24.6†
" G ₃	Ascending	3.4	3.3	6.3	4.6				4.35‡
" G ₂ + G ₃	"	10.4	10.4	10.0	13.9	8.4	9.8	12.3	4.67‡
	Descending	12.7	10.4	16.5	14.2	9.1	10.1		11.18†
	Average	11.5	10.4	13.2	14.1	8.7	9.9		10.5‡
Ovomucoid X	Ascending	4.3	4.2	5.5	3.6			22.1	2.65
	"	2.1	1.9	2.4	3.2			9.3	30†
	Descending	2.3	1.6	3.2	5.3			11.9	10.3†
Ovomucoid + X	Average	2.2	1.8	2.8	4.2			8.5	10.8†
	Ascending	6.4	6.2	7.7	6.8	9.4	9.3		3.45‡
	Descending	7.9	10.6		11.4	9.3	10.5		
Albumin A ₂	Average	7.2	8.4		9.1	9.4	9.9		
	Ascending	12.7	12.4	11.5	13.6			8.4	1.66
	Descending	14.9	12.7	13.8	17.7			7.4	8.48†
" A ₁	Average	13.8	12.6	12.7	15.6			8.5	8.59†
	Ascending	54.8	54.2	52.4	46.9			6.5	2.79
	Descending	53.4	52.5	50.8	42.9			5.9	5.03‡
" A ₁ + A ₂	Average	54.1	53.4	51.6	44.9			5.0	5.43‡
	Ascending	67.5	66.6	63.9	60.5	60.7	65.1	4.0	2.07
	Descending	68.3	65.3	64.6	60.6	64.0	64.3	4.0	2.53
	Average	67.9	65.9	64.3	60.5	64.9	64.7	2.8	2.72

* Three runs each in the case of the first four lots, two each in Groups BAI-A and BAI-B.

† Significant at 1 per cent level.

‡ Significant at 5 per cent level.

Genetic Differences in Chicken—Six groups of chickens of widely different ancestry were chosen to determine what influence inheritable characteris-

tics might have on the protein composition:² Group 16A black australorp, Group 16B Rhode Island red, Group 1 white Leghorn (inbred line No. 8), Group 5 white Leghorn, Group BAI-A non-deteriorating egg strain, and Group BAI-B deteriorating egg strain. In the case of each of the first four groups, the whites of one dozen eggs, obtained by random sampling of a morning's production of thirty-five to forty birds, were pooled and blended together. Three electrophoresis runs were made on subsequent days on each pooled sample. The samples from the last two groups were obtained by pooling the whites of two eggs from each of twelve to fifteen birds. Two runs were carried out on each of these groups. This random selection and pooling should eliminate any individual variations. The results are shown in Table III. An analysis of variance (5) was carried out to determine whether any significant differences exist among the six groups. The ratio of the variance between groups to that within groups, F , is given in the last column. The application of this test criterion results in evidences of significant differences in the globulins, in spite of the fact that resolution in this region is difficult. These differences are not great enough to be of importance in fractionation procedures but point to another characteristic of the avian egg which may be gene-controlled.

SUMMARY

Egg white can be analyzed electrophoretically at pH 7.8 without removal of ovomucin. The identity of the various components is discussed; ovomucin does not manifest itself in the patterns. The influence of ionic strength on mobility of the various components is examined. Data from thirty-one runs under similar conditions are presented and analyzed statistically.

Slight but statistically significant differences are demonstrated in the protein composition of egg white from six genetic strains of chickens.

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² Eggs from the first four groups of chickens were supplied through the courtesy of Dr. A. W. Nordskog of the Iowa State College Department of Poultry Husbandry. The two groups of eggs with the non-deteriorating and deteriorating thick white were supplied through the generosity of the United States Department of Agriculture, Agricultural Research Center, Beltsville, Maryland. The non-deteriorating eggs have a thick white which retains its structure during storage at high temperatures.



EGG WHITE PROTEINS

II. AN ETHANOL FRACTIONATION SCHEME*

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Egg white as a protein system possesses much interest, both biochemical and technological. Study of the egg white proteins has been handicapped by the difficulty of preparation. The five variant system developed by Cohn and coworkers for the fractionation of plasma offers many advantages over the salting-out techniques (1). Low temperatures are employed to improve protein stability. Since low salt concentrations are employed, prolonged dialyses are avoided. Ethanol can be removed easily and rapidly by lyophilization. The ethanol fractionation procedures are adaptable to commercial operations so that production of valuable by-products is a possibility.

In this study, an attempt has been made to adapt the ethanol techniques to the egg white system. Emphasis has been placed on the development of an over-all scheme and not on the preparation of any single fraction. Purity of individual fractions has been a secondary consideration in order to obtain all the fractions in the highest possible yields.

EXPERIMENTAL

The procedures used in this study were similar to those described for the plasma fractionation system (2). Fresh eggs were secured from the Iowa State College Poultry Farm, the whites separated from the yolks, and the chalazae removed. The white was next blended in a Waring blender equipped with a rheostat (to prevent excessive foaming). A commercial grade of 95 per cent ethyl alcohol was diluted to 50 per cent (by volume) and used for all alcohol additions. Acetate, phosphate, and carbonate buffers were used for pH adjustment and electrophoretic runs. Preparation of acetate and phosphate buffers of known ionic strength was facilitated by the use of the d'Ocagne (3) acetate nomogram and Green's (4) phosphate buffer chart. In all fractionation steps, temperatures were

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maintained within 1–2° of the freezing point of the system. All pH measurements were carried out at 25° with a glass electrode on diluted solutions. The fractions were removed by centrifugation and the supernatant solutions clarified by filtration when necessary. A Sharples continuous super-centrifuge was employed when large volumes of solution with low foaming tendencies were centrifuged.

Electrophoretic analysis of the fractions obtained was carried out as previously described (5). Routine analytical runs were made in phosphate-chloride buffer of pH 7.7 to 7.8, $\Gamma/2 = 0.20$ ($\text{NaCl} = 0.15 \text{ M}$). Averages of ascending and descending data were used in most instances. Nitrogen was determined by a modified Pregl micro-Kjeldahl procedure with SeOCl_2 as a catalyst.

RESULTS AND DISCUSSION

Egg white has been separated into six fractions by application of the ethanol fractionation procedures. The results of a typical fractionation experiment are shown in Figs. 1 and 2 and the data summarized in Tables I and II. 1 liter of egg white, of the following composition, was used as the starting material:¹ nitrogen 17.69, ovalbumin 11.50, ovomucoid 1.64, globulin 1.54, conalbumin 2.41, and lysozyme 0.60 gm. In Figs. 1 and 2, the conditions imposed on the system are given on the horizontal tie lines where N_2 is the mole fraction of ethyl alcohol and N is the nitrogen in the system in gm. per liter. The analytical data for each fraction are given in the boxes where N is the total gm. of nitrogen in the fraction and A , O , G , C , and L represent the gm. of ovalbumin, ovomucoid, globulin, conalbumin, and lysozyme nitrogen, respectively. No attempt was made to convert the nitrogen data to a protein basis.

The separation and purification of each fraction are discussed in the order in which they were removed from the egg white.

Ovomucin Fraction—Egg white, blended in a Waring blender, was subjected to high speed centrifugation in a Sharples air-driven supercentrifuge equipped with a batch type bowl. At a velocity of 45,000 r.p.m. (50,000 $\times g$), a slimy, ropy precipitate characteristic of ovomucin was removed after 15 minutes centrifugation. This fraction was designated Fraction I. In several experiments, 1.1 per cent of the original protein (by N analysis) was removed by this procedure. Attempts to remove ovomucin by the classical dilution or salting out techniques resulted in the precipitation of approximately 10 per cent of the protein, while the procedure used in this study effected the separation of only 1.1 per cent of the original nitrogen. This figure is probably more reliable than the higher figure (1.9 per cent)

¹ Data obtained by electrophoretic analysis; ovomucin included only in the figure for total nitrogen.

previously reported (6) because of the extreme difficulty of washing soluble proteins from the classical ovomucin precipitate. That ovomucin can be removed by centrifugation without any previous treatment supports the view that this protein is present in egg white in the form of very large (fibrillar) particles.

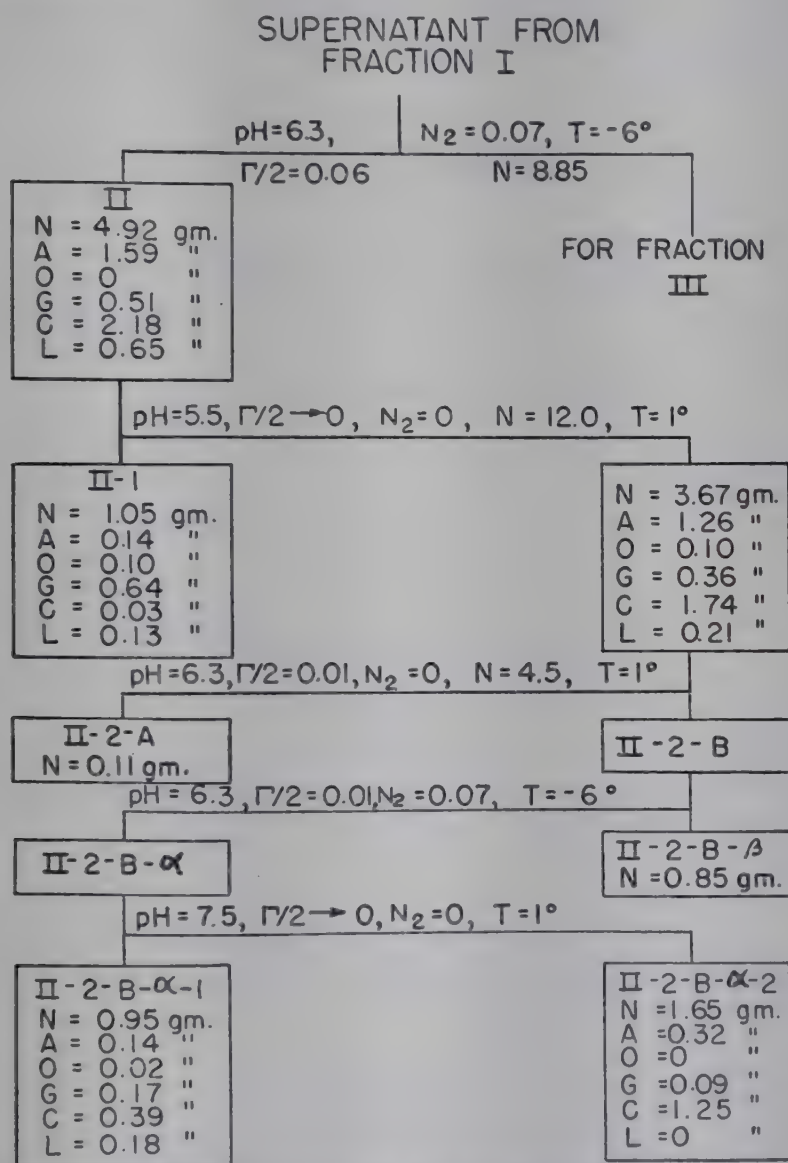


FIG. 1. Separation and purification of the conalbumin-globulin fraction. For an explanation of the symbols, see the text.

The precipitate as removed from the centrifuge rotor can be suspended by means of the Waring blender in 0.15 M (or higher) NaCl to yield reasonably stable, though highly turbid, suspensions. Many other solvent systems have been tried, including urea (6 M) and both anionic and cationic detergents at pH levels as high as 12, none of them yielding anything ap-

proaching clear solutions. On the other hand, this precipitate can be blended with egg white to give solutions practically as clear as the original white. This may arise from the solvent action of the other proteins or may be a refractive index effect.

Conalbumin-Globulin Fraction—Bain and Deutsch (7) described an ethanol fractionation scheme for the preparation of conalbumin from egg white. A preparation of high purity was obtained, although in low yield.

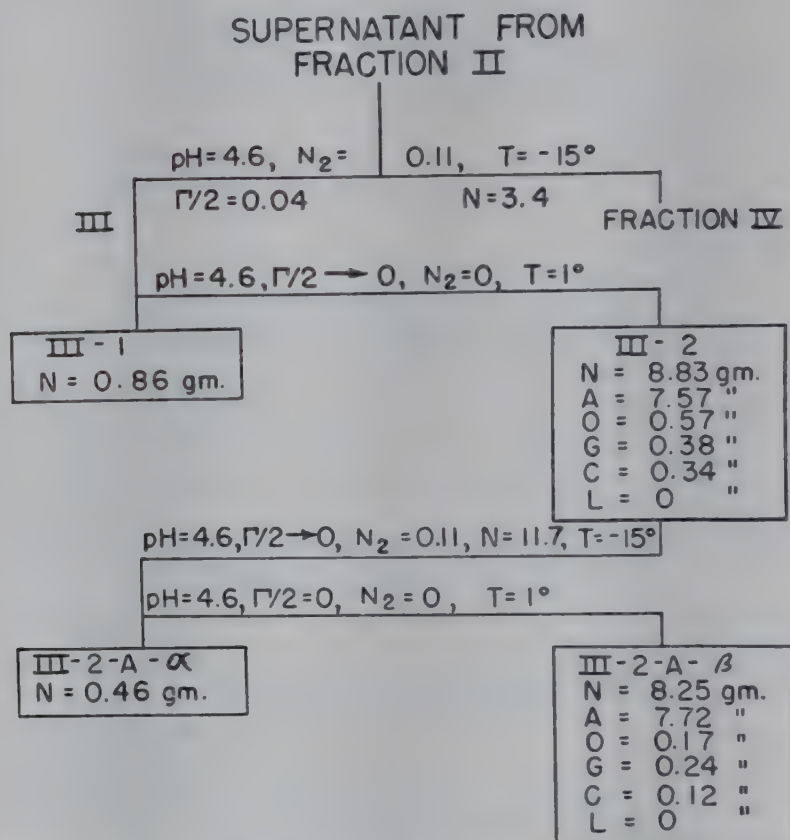


FIG. 2. Separation and purification of the ovalbumin fraction. For an explanation of the symbols, see the text.

In this investigation, it has not been possible to obtain satisfactory separation of either the conalbumin or globulin fraction individually. Several attempts were made to remove the globulin prior to the conalbumin at pH 5.4 to 5.6 and low ionic strengths. In all cases, the fraction was highly contaminated with conalbumin and considerable quantities of apparently denatured protein were obtained. This procedure was not considered theoretically sound because of the presence of oppositely charged proteins, a condition which usually results in extensive interactions. Attempts to remove conalbumin at pH 6.0 to 7.0, $\Gamma/2 = 0.05$ to 0.10 , $N_2 = 0.02$ to 0.04 , were unsuccessful because of large amounts of globulin im-

purities and incomplete precipitation of the conalbumin; 15 to 25 per cent of the total globulin was precipitated under these conditions. The con-

TABLE I
Conditions for Separation of Egg White Proteins into Fractions

Fraction No.	pH	$\frac{I}{2}$	<i>T</i>	Ethanol, N ₂	N	N in fraction	N of original
			°C.		gm. per l.	gm.	per cent
I.....	Supercentrifuge				17.69	0.20	1.1
II.....	6.3	0.06	-6	0.07	8.85	4.92	27.8
II-1.....	5.5	→0	1	0	12.00	1.05	5.9
II-2-A.....	6.3	0.01	1	0	4.5	0.11	0.6
II-2-B-β.....	6.3	0.01	-6	0.07		0.85	4.8
II-2-B-α-1.....	7.5		1	0		0.95	5.4
II-2-B-α-2.....	7.5		1	0		1.65	9.3
III.....	4.6	0.04	-15	0.11	3.4	9.69	54.6
III-1.....	4.6	→0	1	0		0.86	4.8
III-2-A-α.....	4.6	→0	1	0		0.46	2.6
III-2-A-β.....	4.6	→0	1	0		8.25	46.7
IV.....	Supernatant		1	0		0.54	3.1
Total yield.....							84.3

N₂ is the mole fraction of ethanol.

TABLE II
Distribution of Egg White Proteins into Fractions

Fraction No.	Ovalbumin		Ovomucoid		Globulin		Conalbumin		Lysozyme	
	(1)*	(2)†	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Egg white.....	64.9		9.2		8.7		13.8		3.4	
II-1.....	13.4	1.2	9.5	6.1	61.0	41.5	2.9	1.2	12.4	21.7
II-2-B-α-1.....	14.7	1.2	2.1	1.2	17.9	11.0	41.0	16.2	19.0	20.0
II-2-B-α-2.....	19.4	2.8	0	0	5.4	5.8	75.8	52.0	0	0
III-2-A-β.....	93.7	67.2	2.1	10.4	2.9	15.5	1.5	5.0	0	0
IV.....	11.1	0.5	88.9	29.3	0	0	0	0	0	0
Total yield.....	72.9		47.0		73.8		74.4		41.7	

* (1), per cent of constituent in the fraction.

† (2), per cent of constituent, in the fraction, of that originally present in the egg white.

albumin-globulin fraction was satisfactorily removed as Fraction II under the conditions described in Fig. 1. There was no sharp pH optimum

between pH 6.0 to 7.0 for the removal of this fraction, but somewhat better removal was effected in the more acid range. This fraction was removed by supercentrifugation, and, although attempts were made to complete the removal without excessive temperature increases, the effluent from the centrifuge was usually 5–7° higher than that at which the fraction had been precipitated. Upon cooling the solution to the precipitation temperature, a precipitate formed, indicating a high temperature coefficient of solubility of the proteins in this medium. Repeated cooling and centrifugation, followed by filtration at the precipitation temperatures, apparently effected complete removal of the fraction. Only about 50 per cent of the globulin can be removed under these conditions, indicating the presence of at least two globulin fractions with different solubility characteristics. The globulin that remains in solution appears to be similar to the pseudoglobulin reported by Kuchel and Bate-Smith (8). A partial separation of the globulins was accomplished by these procedures.

All of the lysozyme present in the egg white is removed in this fraction and can be crystallized by the procedure described by Alderton and Fevold (9). Several fractionations have been carried out in which lysozyme has been removed prior to the conalbumin-globulin fraction. Yields of lysozyme are higher under these conditions, but the fractionation procedure is prolonged by the extended crystallization period and all of the proteins are subjected to high pH and salt concentrations. The removal of the salt before further fractionation is a tedious and time-consuming process. For best results in a comprehensive fractionation scheme, lysozyme should be removed with the conalbumin-globulin fraction and then recovered by sub-fractionation.

The globulin was separated from the conalbumin by lowering the pH to 5.5 and dialyzing against water to lower the ionic strength. The degree of separation obtained depends on the thoroughness of the washing of the precipitate. All of the yellow color of the egg white was found in the conalbumin (Fraction II-2, Fig. 1). Further fractionation of Fraction II-2 resulted in two main fractions (Fractions II-2-B- α -1 and II-2-B- α -2). Although Fraction II-2-B- α -2 contained a considerably higher proportion of conalbumin, Fraction II-2-B- α -1, insoluble in water but soluble in 5 per cent NaCl, contained all of the yellow constituent originally present in the fraction. This was unexpected, since it had been shown (7) that the riboflavin is bound to the conalbumin and can be removed by dialysis only on the acid side of the isoelectric point. It was concluded that either (1) the riboflavin is not bound to the characteristic conalbumin constituent, (2) the conalbumin does not behave as a typical albumin, or (3) the bound riboflavin appreciably alters the solubility properties of conalbumin.

Ovalbumin Fraction—Following removal of the conalbumin-globulin frac-

tion, ovalbumin was removed as Fraction III by lowering the pH to 4.6 and increasing the ethanol concentration to mole fraction 0.11, as shown in Fig. 2. All of the protein remaining after the removal of Fraction II, except ovomucoid, was removed nearly quantitatively under these conditions. Purification of the ovalbumin was carried out as indicated in Fig. 2. Typical analytical data are shown in Table II. The ovalbumin exhibits a high temperature coefficient of solubility in this medium, as do the conalbumin and globulin. The solubility was lowered as the NaCl concentration was decreased in qualitative agreement with the observations of Ferry, Cohn, and Newman (10). The initial removal of this fraction might be improved by reducing the ionic strength to approximately 0.01, since protein-protein interactions would probably be of minor importance in this case.

The ovalbumin from this fraction was readily crystallized by slight modification of the classical ammonium sulfate procedures (11) and was soluble in water at its isoelectric point. Attempts to crystallize the ovalbumin from alcohol-water according to the procedures described by Cohn, Hughes, and Weare (12) for serum albumin have resulted in small yields of crystalline material, not yet demonstrated to be protein.

Ovomucoid Fraction—In the present procedure, the ovomucoid has been concentrated in the supernatant remaining after the removal of the ovalbumin fraction. As the purity of this fraction (IV), judged by electrophoretic analysis, has been quite high, no attempt has been made to precipitate ovomucoid from it. The volume of the supernatant containing the ovomucoid has been about 3 to 4 times the volume of the initial egg white. Concentration has been effected by pervaporation from large tubular Visking membranes, alcohol and salts being subsequently removed by dialysis and dry ovomucoid preparations obtained by lyophilization. Yields of ovomucoid were usually higher than that given in Table II; in several experiments, 80 to 85 per cent of the ovomucoid was recovered in fractions which consisted of 75 to 80 per cent ovomucoid. The major impurity in all cases was ovalbumin, which was readily removed by repetition of the ovalbumin precipitation. Ovomucoid preparations obtained in this study have not been tested for antitryptic activity. In electrophoretic experiments, ovomucoid has exhibited reversible boundary spreading as described by Longsworth *et al.* (13), indicating inhomogeneity.

SUMMARY

1. A method for the comprehensive fractionation of egg white in media of low dielectric constant and low ionic strength is described. Several advantages are claimed for this method over those previously reported for preparing egg white fractions.

Ovomucin has been removed by supercentrifugation with a minimum of alteration and occlusion of other proteins.

The ethyl alcohol acts as a foam depressant so that the Sharples supercentrifuge can be used for continuous operation.

The advantages previously described for the preparation of the plasma proteins are applicable to the egg white fractionation.

2. The possibility that the riboflavin of egg white is not bound to the main conalbumin component is suggested.

3. Evidence is given to support the view that two globulins, with different solubility characteristics, are present in egg white.

4. Ovalbumin prepared by these procedures is readily crystallized by the use of ammonium sulfate.

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PREPARATION OF DEUTERIUM STEROID HORMONES FROM BILE ACIDS AND THE FORMATION OF α,β -UNSATURATED KETONES FROM 3-KETOCHOLANE DERIVATIVES*

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As a portion of a broad program dealing with the metabolism of steroids, we wished to prepare hormones with deuterium in a known stable position in the molecule. The reactions leading to the preparation of progesterone and testosterone are described in this report, since certain problems of general interest have been encountered and their solution is important in other areas of steroid research. An extension of the investigation has been briefly reported (1) and further results will be described subsequently.

As one possible means for the preparation of these steroid hormones, methyl 3α -acetoxy- Δ^{11} -cholenate (2) was reduced with deuterium and by a known series of reactions (3) was converted to pregnanolone (pregnan- 3α -ol-20-one), with 1.65 deuterium atoms per mole of compound. Two principal problems in the conversion of pregnanolone to progesterone and testosterone were the oxidation of a C_{21} steroid to a C_{19} compound and the introduction of the 4,5-double bond in the intermediate saturated 3-ketone. The latter problem was critical, since it was encountered at the end of a series of reactions in which loss of valuable material in a reaction poor with respect to yield was especially discouraging.

Oxidation of C_{21} to C_{19} Steroids—Although a variety of methods could have been used to effect this transformation, one of the most generally satisfactory procedures both with respect to ease of manipulation and yield of product was the perbenzoic acid oxidation of a suitable derivative of a 20-ketosteroid (4-6). Although the yield of 17-acetoxy steroid was seldom higher than 50 per cent, a considerable portion of unchanged material could be recovered and recycled. The C-3 hydroxyl group was pro-

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tected by the formate ester, because differential removal by adsorption on a suitable alumina (7) was readily possible, whereas only a minor difference in rate of saponification could have been anticipated between a 3α and a 17β acetoxy group.

Oxidation of C-3 alcohols to keto groups in these saturated compounds was most readily accomplished by means of *N*-bromoacetamide (8). This reaction proceeded smoothly and gave higher yields than the more commonly used CrO_3 oxidation.

The introduction of the 4,5 double bond in the 3-ketocholane derivatives was achieved through the 4-bromo ketone as an intermediate, followed by dehydrobromination to the α,β -unsaturated ketones. The classical dehydrobromination with pyridine or collidine (9) which has been widely used is known to give generally unsatisfactory yields (10). Substantially better results with a new method have been reported by Mattox and Kendall (11, 12) who found that the 2,4-dinitrophenylhydrazones of 4-bromo ketones of the normal series were brominated very readily and the derivatives could be cleaved in good yield to the desired α,β -unsaturated ketones. Djerassi has confirmed these results (13) and applied the method to bromo ketones of the allo series; he likewise reported the use of other ketone derivatives. Because of certain difficulties encountered in following these procedures, we undertook a critical study of these dehydrobromination reactions which led to the following observations and improvements.

Bromination of 3-Ketocholane Derivatives—It was soon apparent that rigorous purification of the 4-bromo ketone was essential in order to obtain a uniform dehydrobromination product in good yield. It was found that 3-ketocholane derivatives upon bromination with 1 mole of bromine yielded a crystalline product which in addition to the 4-bromo ketone contained a variable amount of a dibromo ketone. The presence of the dibromo ketone could be recognized from the optical rotation which was lowered by the more levorotatory side product. The melting point was an unsatisfactory criterion for purity in the instances examined, since the melting points of the bromo ketones are in the same range and were not depressed upon admixture.

The constitution of the isolated side products can be inferred from the elementary analysis and from the infra-red absorption spectra. Infra-red analysis revealed a shift of the 3-ketone band from 1719 to 1758 cm.^{-1} . In several examples which have been studied in this Institute by Jones, Humphries, and Dobriner (14) monobromo 3-ketones of both the normal and allo series have exhibited a shift of the carbonyl absorption to 1735 cm.^{-1} . When two halogens were on the same carbon, the carbonyl absorption was also at 1735 cm.^{-1} . The fact that the carbonyl absorption is shifted to the same wave-length by bromine substitution in both the

normal and allo series, whether 1 or 2 bromine atoms are situated on the same carbon atom, provides good evidence that the shift to 1758 cm.^{-1} is a consequence of bromine substitution on two different carbon atoms α to the 3-ketone group.

Further support for the correctness of these conclusions is provided by the examination of four examples (15) of authentic 2,4-dibromo ketones of the allo series in which the carbonyl absorption was found between 1758 and 1756 cm.^{-1} . It seems quite reasonable therefore to assume that the products we have isolated are 2,4-dibromo ketones of the normal series, and we have therefore so designated these compounds. It is realized that definite chemical proof should be provided, but this is beyond the scope of the present report. The generally applicable procedure we have established is to select for dehydrobromination the fractions exhibiting the greatest dextrorotation together with the characteristic infra-red absorption at 1735 cm.^{-1} and to reduce and recycle the remaining product.

Dehydrobromination Reaction—With a pure 4-bromo-3-ketocholane derivative, dehydrobromination could best be achieved by a modification of the Mattox and Kendall procedure; the dinitrophenylhydrazone after formation was readily cleaved by heating with pyruvic acid without isolation of the intermediate. The addition of hydrobromic acid to the reaction mixture proved not only unnecessary but actually disadvantageous, since pyruvic acid was destroyed and a 20-ketone was isomerized. Several alterations of the reaction and variations in the experimental conditions were studied and these are described in the experimental section. Yields of pure α,β -unsaturated ketone exceeding 80 per cent could be obtained with the method we have chosen without spectroscopic evidence of the formation of a $\Delta^{4,6}$ -dienone. Mattox and Kendall (12) have reported that autoxidation of a dinitrophenylhydrazone and dehydrobromination of a polybromo ketone can yield a $\Delta^{4,6}$ -dienone. The modified Mattox-Kendall procedure we have used does not effect dehydrobromination of the two 2,4-dibromo-3-ketocholane derivatives studied and the products obtained after cleavage of the pure monobromo ketones did not show spectroscopic evidence of a $\Delta^{4,6}$ -dienone. The 2,4-dibromopregnane-3,20-dione was recovered unchanged in our experiments, even after three successive treatments with dinitrophenylhydrazine followed by cleavage with pyruvic acid. This result is interesting in the light of Djerassi's observation (13) that 2,4-dibromo-3-ketoallosteroids yield a mixture of $\Delta^{1,4}$ -dien-3-dinitrophenylhydrazone and a product containing approximately 1 bromine atom, and that neither product could be cleaved with pyruvic acid by the Mattox-Kendall method.

Equally satisfactory results to those with dinitrophenylhydrazine could be obtained with semicarbazide (13). The dehydrobromination proceeded

readily, and cleavage of the derivative in the absence of strong acid was effected by the excellent procedure of Hershberg (16). When the dehydrobromination of 4-bromopregnane-3,20-dione was attempted with Girard's Reagent T, a water-soluble product resulted, but the derivative could not be cleaved even with high concentrations of mineral acid. The dehydrobromination of pure 4-bromopregnane-3,20-dione with pyridine was studied for comparison with the other procedures. The yield of progesterone, although inferior to that obtained from the dinitrophenylhydrazones and semicarbazones, exceeded that obtained by previous investigators (10). With impure bromo ketone, the yield of progesterone was proportional to the amount of pure 4-bromopregnane-3,20-dione calculated from the rotation to be present in the crude product. This is consistent with the observations (17, 18) that pyridinium salts of 2,4-dibromo-3-ketosteroids are more stable and less readily pyrolyzed than the pyridinium salts of 4-bromo-3-ketosteroids.

EXPERIMENTAL

Methyl d_2 -11,12-Lithocholate—Methyl 3α -acetoxy- Δ^{11} -cholenate was reduced with 95 per cent deuterium gas in 10 gm. portions by use of 2 gm. of $\text{PtO}_2 \cdot \text{H}_2\text{O}$ in 125 ml. of acetic acid- d . The solution was decanted from the catalyst and filtered quickly; the same catalyst was used to reduce about 150 gm. of product. The acetic acid- d was distilled in a closed system under diminished pressure and was used repeatedly. The accumulation of atmospheric water in repeated transfers, filtrations, etc., and the use of 95 per cent deuterium gas account for the incorporation of less than the calculated amount of deuterium. When rigid precautions are taken to eliminate these dilutions, the incorporation of the isotope is usually slightly in excess of theory. The methyl acetoxy- d_2 -11,12-lithocholate was saponified with N NaOH under a reflux for 1 hour, and the acid was esterified with methanol in the presence of H_2SO_4 at room temperature. The product contained 4.03 atoms per cent excess deuterium (1.69 atoms of deuterium per mole).

3α -Hydroxy- d_2 -11,12-pregnan-20-one was obtained from methyl d_2 -11,12-lithocholate by the procedure of Meystre and Miescher (3). The product had the following constants: m.p. 150 – 151° , $[\alpha]_D^{25} = +117^\circ$ (CHCl_3), 4.89 atoms per cent excess deuterium or 1.64 atoms of deuterium per mole. Another crystalline modification of pregnanolone was obtained which melted at 132 – 133° ; $[\alpha]_D^{25} = +117^\circ$ (CHCl_3). The infra-red spectra of these two crystalline forms were identical in solution but different in suspension in mineral oil.

17β -Acetoxy- d_2 -11,12-etiocolan- 3α -ol. From Pregnanolone—250 mg. of d_2 -11,12-pregnanolone were treated with 1.8 ml. of 0.87 M perbenzoic acid

in carbon tetrachloride at room temperature, approximately 25°, for 8 days. All the perbenzoic acid had disappeared during this interval. The reaction product was isolated in the usual way. 35 mg., m.p. 217–218.5°, $[\alpha]_D^{25} = +34^\circ$ (CHCl_3), of 17 β -acetoxy-4-hydroxy secoetiocholan-3 acid lactone were obtained (saponification equivalent 345). Infra-red analysis showed the absence of a free hydroxyl group.

$\text{C}_{21}(\text{H} + \text{D})_{32}\text{O}_4$ (350.09). Calculated. C 72.04, H + D 9.68
Found. " 71.86, " + " 9.38

The mother liquors (241 mg.) were separated into ketonic (161 mg.) and non-ketonic (80 mg.) fractions by means of Girard's Reagent T. Crystalline 17 β -acetoxy-*d*₂-11,12-etiocholan-3 α -ol, m.p. 168–170°, was obtained from the non-ketonic fraction. Saponification yielded etiocholane-3 α ,17 β -diol; m.p. 235–235.5°, $[\alpha]_D^{25} = +22.9^\circ$ (ethanol).

From 3 α -Formoxy-*d*₂-11,12-pregnan-20-one—1.0 gm. of *d*₂-11,12-pregnanolone was dissolved in 4 ml. of formic acid, specific gravity 1.2, and heated at 70° for 4.5 hours. The excess formic acid was removed under reduced pressure and the product showed the absence of free hydroxyl groups by infra-red analysis. Without crystallization, the product was dissolved in 1 ml. of chloroform, and 3.38 ml. of 0.87 M perbenzoic acid in chloroform were added. After standing at 25° for 11 days, no perbenzoic acid remained, and 1.027 gm. of an oily product were obtained in the usual manner. Separation with Girard's Reagent T yielded 595 mg. of crystalline product in the non-ketonic fraction and 406 mg. in the ketonic fraction. The non-ketonic fraction was adsorbed upon 100 times its weight of an aluminum oxide known to be capable of hydrolyzing esters and allowed to stand upon the column for 24 hours. Elution was effected with ether-methanol and, after two recrystallizations from acetone-petroleum ether, 382 mg. of 17 β -acetoxy-*d*₂-11,12-etiocholan-3 α -ol were obtained; m.p. 169.5–170°, $[\alpha]_D^{28} = +18.2^\circ$ (CHCl_3). The infra-red spectrum showed the presence of an acetoxy group and a free hydroxyl.

$\text{C}_{21}(\text{H} + \text{D})_{34}\text{O}_4$ (336.10). Calculated. C 75.04, H + D 10.68
Found. " 74.96, " + " 10.58

*d*₂-11,12-Pregnane-3,20-dione. CrO_3 Oxidation—505 mg. of 3 α -hydroxy-*d*₂-11,12-pregnan-20-one, m.p. 148–151°, were dissolved in 10 ml. of glacial acetic acid which had been redistilled from CrO_3 . 140 mg. (2.2 equivalents) of CrO_3 dissolved in 34 ml. of acetic acid were added and after 20 hours at 15° the excess CrO_3 was reduced with ethanol. The acetic acid was evaporated under reduced pressure and the residue was dissolved in ether and washed with 5 per cent sodium carbonate solution and with water. The ether solution was dried over anhydrous sodium

sulfate, and after removal of the solvent, 382 mg. of pregnanedione, m.p. 119–121°, $[\alpha]_D^{16} = +105^\circ$ (CHCl_3), crystallized from ether-petroleum ether. Chromatography of the mother liquors upon Al_2O_3 yielded an additional 38 mg. of the same purity. The total yield was 84 per cent.

N-Bromoacetamide Oxidation—600 mg. of 3α -hydroxy- d_2 -11,12-pregnan-20-one were dissolved in 3 ml. of *tert*-butanol and 0.9 ml. of pyridine, 4 drops of water, and 500 mg. of *N*-bromoacetamide (4 equivalents) were added. The reaction mixture was stored in a stoppered flask overnight at 25°. The dark solution was dissolved in ether and water and the ether solution washed thoroughly with dilute sodium bicarbonate, with hydrochloric acid, and with water. The ether was dried with anhydrous sodium sulfate and the solvent removed under diminished pressure. The residue was thoroughly dried and, after extraction with dry ether, most of the dark impurities remained undissolved. The ether-soluble product was dissolved in 1:9 benzene-petroleum ether and chromatographed on a column containing 10 times its weight of Al_2O_3 . Elution was accomplished with 1:1 benzene-petroleum ether and the product was crystallized from ethyl acetate-petroleum ether. 500 mg. of pregnanedione, m.p. 120–122°, were obtained. Chromatography of the mother liquors afforded an additional 50 mg. of the same purity. The total yield was 90 per cent.

4-Bromo- d_2 -11,12-pregnane-3,20-dione—640 mg. of d_2 -11,12-pregnane-3,20-dione in glacial acetic acid were chilled in an ice bath and 4.5 ml. of a 0.49 M solution of bromine in glacial acetic acid (2.10 equivalents) were added dropwise within 20 to 30 minutes. The rate of addition was determined by the decolorization of the bromine previously added. The acetic acid was evaporated to a few ml. at 40° under reduced pressure and addition of ether yielded a crystalline product. After filtration and recrystallization from chloroform-ether, 410 mg. (51 per cent) of hexagonal prismatic needles, which rearranged partially at 175° and melted with decomposition at 180–184°, $[\alpha]_D = +106^\circ$ (CHCl_3), were obtained.

$\text{C}_{21}(\text{H} + \text{D})_{31}\text{O}_2\text{Br}$ (397.01). Calculated, Br 20.13; Found, Br 20.31

From the combined mother liquors 120 mg. (15 per cent) of hexagonal crystals were obtained by crystallization from chloroform-ether. This second fraction melted at 175–180° and gave no depression upon admixture with the crystals from the first fraction, although the rotation was markedly lower; $[\alpha]_D = +83^\circ$ (CHCl_3). Its further purification is described below.

The remaining mother liquors (200 mg.), $[\alpha]_D = +22^\circ$, representing mixtures of lower melting and amorphous products, were reconverted to pregnanedione by solution in 2 ml. of glacial acetic acid and heating for 1 hour at 80° with 200 mg. of zinc dust. The product was isolated as

previously described. 100 mg. of pure d_2 -11,12-pregnane-3,20-dione (16 per cent based on the initial amount of pregnanedione) were recovered.

2,4-Dibromo- d_2 -11,12-pregnane-3,20-dione—The second crystalline fraction, m.p. 175–180°, $[\alpha]_D = +83^\circ$, was repeatedly recrystallized from chloroform-ether mixtures. The more dextrorotatory fractions were removed and the products with lower rotations were purified. 25 mg., $[\alpha]_D = +70.5^\circ$, were thus isolated as hexagonal prisms, m.p. 183–185°; the rotation remained constant on further recrystallization.

$C_{21}(H + D)_{30}O_2Br_2$ (475.91). Calculated, Br 33.59; found, Br 31.78

When mixed with pure 4-bromo- d_2 -11,12-pregnane-3,20-dione, $[\alpha]_D = +106^\circ$ and m.p. 180–184°, there was no depression of the melting point.

d_2 -11,12-Progesterone and d_2 -11,12-17 α -Progesterone (Mattox-Kendall Procedure)—135 mg. of 4-bromo- d_2 -11,12-pregnane-3,20-dione, m.p. 178–184°, and 75 mg. of 2,4-dinitrophenylhydrazine were dissolved in 5 ml. of glacial acetic acid and heated for 15 minutes at 60° under nitrogen. The 2,4-dinitrophenylhydrazone was precipitated with 15 ml. of 5 per cent hydrochloric acid, filtered, washed with 5 per cent hydrochloric acid and with water, and dried in a vacuum. It was redissolved in a small amount of chloroform and reprecipitated and washed with methanol. 160 mg. were obtained.

To 160 mg. of 2,4-dinitrophenylhydrazone in 6 ml. of chloroform, 8 ml. of 85 per cent pyruvic acid, 0.1 ml. of water, and 0.8 ml. of 3.21 N hydrobromic acid in glacial acetic acid were added. The mixture was sealed under nitrogen and heated to 60°, and became monophasic at this temperature. The color changed from red to yellow in from 2 to 2.5 hours. The solution was transferred with about 50 cc. of ether to a separatory funnel, extracted with enough sodium carbonate solution to remove all of the pyruvic acid-dinitrophenylhydrazone, washed twice with 5 per cent sodium hydroxide and several times with water, and dried over anhydrous sodium sulfate. The residue from the ether solution was still colored, and crystallization could be initiated only by inoculation. 110 mg. were obtained which, after sublimation in a high vacuum at 90–100°, gave 104 mg. of sublimate which was chromatographed upon a column of 10 gm. of alumina. Benzene eluted 63 mg. which were recrystallized and identified as d_2 -11,12-progesterone, m.p. 120–123°, $[\alpha]_D = +180^\circ$ (acetone), $\epsilon_{2410} = 16,800$ (methanol). The yield was 50 per cent. Subsequent elution of the chromatogram with benzene-ether mixtures yielded 10 mg. of crystals (10 per cent) which were identified as d_2 -11,12-17 α -progesterone, m.p. 143–146°, $[\alpha]_D = +9^\circ$ (acetone), $\epsilon_{2410} = 16,400$ (methanol). Butenandt *et al.* reported for this compound $[\alpha]_D = 0^\circ$, a melting point of 145°, and $\epsilon_{2410} = 16,300$ (19).

Dehydrobromination of Pure 4-Bromo- d_2 -11,12-pregnane-3,20-dione with Pyridine—70 mg. of the bromo ketone with $[\alpha]_D = +106^\circ$ were dissolved in 3 ml. of pure pyridine, sealed under nitrogen, and heated for 18 hours at 130° . The solution was diluted with ether and washed with 5 per cent hydrochloric acid, with 5 per cent sodium carbonate, and with water. The aqueous washings were reextracted twice with ether. The residue, 43 mg. (77 per cent of the calculated yield), crystallized but was not pure progesterone. Chromatography yielded 27 mg. (50 per cent) of pure progesterone together with two unidentified products which differed in rotation. Upon crystallization the early fractions eluted with 10 to 20 per cent benzene in petroleum ether gave 5 mg. with a melting point of $95\text{--}120^\circ$, $[\alpha]_D = +137^\circ$ (CHCl_3). The late fractions eluted with 5 to 10 per cent ether in benzene gave 5 mg. with a melting point of $135\text{--}140^\circ$ $[\alpha]_D = +30^\circ$ (CHCl_3).

90 mg. of crude bromo- d_2 -11,12-pregnane-3,20-dione, $[\alpha]_D = +83^\circ$, were treated with pyridine as described in detail for the pure bromo ketone, $[\alpha]_D = +106^\circ$. 38 mg. (55 per cent) were recovered from which 20 mg. of progesterone, m.p. $117\text{--}123^\circ$, and a small amount of more contaminated material were isolated.

d_2 -11,12-Progesterone (Modified Procedure)—The following procedure was found to be the best and most convenient. 100 mg. of the purest specimen of 4-bromo- d_2 -11,12-pregnane-3,20-dione, $[\alpha]_D = +106^\circ$, were dissolved in 3 ml. of glacial acetic acid at 60° and 25 mg. of sodium acetate were added. 60 mg. of 2,4-dinitrophenylhydrazine (1.2 equivalents) were added and dissolved under a stream of nitrogen. The reaction mixture was kept at 60° for 20 minutes, during which the mono-2,4-dinitrophenylhydrazone of progesterone crystallized. To the suspension, 5 ml. of chloroform, 50 mg. of sodium acetate, 3 ml. of freshly distilled pyruvic acid, and 1 ml. of water were added. The resulting dark red solution was refluxed under a stream of nitrogen at about 70° until the cleavage of the dinitrophenylhydrazone was completed. This was recognized by the color change from red to yellow and was achieved within 3 hours. During this period, 3 ml. of pyruvic acid and 2 ml. of water were added in three portions as the reaction proceeded. The product was isolated as previously described. 78 mg. of progesterone were crystallized from ether-petroleum ether without inoculation. The crude product was sublimed at $90\text{--}100^\circ$ in a high vacuum and was thus freed from persistent colored impurities. Recrystallization from ether-petroleum ether gave 64 mg. of pure progesterone melting at $127\text{--}129^\circ$; $[\alpha]_D = +200^\circ$ (CHCl_3), $\epsilon_{2410} = 16,800$ (methanol), 5.58 atoms per cent excess deuterium (1.67 atoms of deuterium per mole). No d_2 -11,12-17 α -progesterone was obtained under these experimental conditions. The yield was 80 per cent.

Experimental Variations of Mattox-Kendall Procedure—Formation of the

bis-2,4-dinitrophenylhydrazone from 4-bromopregnane-3,20-dione did not improve the yield of progesterone. This intermediate proved to be less soluble and less easily split than the monodinitrophenylhydrazone. 330 mg. of 4-bromopregnane-3,20-dione yielded 520 mg. (97 per cent) of progesterone-bis-dinitrophenylhydrazone. This required 50 ml. of chloroform for solution and was cleaved after 4.5 hours of treatment. The yield of progesterone was 200 mg. or 75 per cent.

Absence of Sodium Acetate—Sodium acetate was not required for the formation and dehydrobromination of the dinitrophenylhydrazone. 300 mg. of 4-bromopregnane-3,20-dione yielded 190 mg. or 80 per cent of pure progesterone. *Isolation of dinitrophenylhydrazone* and purification of this did not offer any advantage, as judged by the purity and the final yield of progesterone. 135 mg. of 4-bromopregnane-3,20-dione yielded 180 mg. of monodinitrophenylhydrazone which was precipitated from acetic acid with 5 per cent HCl in water, filtered, washed with water, dried in a vacuum, redissolved in chloroform, and reprecipitated with methanol. After cleavage, 80 mg. or 75 per cent of progesterone were obtained.

Mattox-Kendall Reaction with Bromo- d_2 -11,12-pregnane-3,20-dione of $[\alpha]_D = +83^\circ$ —2,4-Dibromo- d_2 -11,12-pregnane-3,20-dione is recovered unchanged after treatment with dinitrophenylhydrazine and pyruvic acid. 135 mg. of the brominated pregnanedione, m.p. $175-180^\circ$, $[\alpha]_D = +83^\circ$, were heated to 70° in 6 ml. of glacial acetic acid with 85 mg. of 2,4-dinitrophenylhydrazine (1.3 equivalents) and 30 mg. of anhydrous sodium acetate for 25 minutes. 189 mg. of dinitrophenylhydrazone which still exhibited a positive Beilstein reaction were isolated. The product was treated with pyruvic acid in acetic acid-chloroform-water for 3 hours at 65° and worked up as described. 40 mg. of a bromo compound crystallized from the dried residue on the addition of ether. This compound melted at $175-180^\circ$ and gave no depression of melting point upon admixture with the initial product. From the mother liquor, progesterone and mixtures of progesterone with the bromo compound were obtained by fractional crystallization. 110 mg. of bromine-containing fractions were again treated in acetic acid with 150 mg. (3 moles) of 2,4-dinitrophenylhydrazine for 40 minutes at 70° in the presence of 30 mg. of sodium acetate under nitrogen. 168 mg. of bisdinitrophenylhydrazone were obtained which were cleaved and worked up as described. The same bromo compound crystallized from ether, yielding 40 mg. with a melting point of $175-180^\circ$. After three recrystallizations from chloroform-ether, 15 mg. of pure product were obtained which, from rotation and infra-red analysis, proved to be identical with the 2,4-dibromo- d_2 -11,12-pregnane-3,20-dione isolated from the starting material by direct crystallization; $[\alpha]_D = +69.5^\circ$ (CHCl_3).

$\text{C}_{21}(\text{H} + \text{D})_{30}\text{O}_2\text{Br}_2$ (475.91). Calculated, Br 33.59; found, Br 33.46

The combined mother liquors from these reactions, 80 mg., were chromatographed on 3 gm. of alumina. Progesterone was obtained from the fractions eluted with 1:9 to 2:3 benzene-petroleum ether. Together with that isolated by direct crystallization the yield amounted to 30 mg. or 33 per cent. No other compound could be isolated from the chromatogram in sufficient quantity for identification.

d₂-11,12-Progesterone. Bissemicarbazone Method—48 mg. of 4-bromo-*d₂-11,12-pregnane-3,20-dione*, $[\alpha]_D = +106^\circ$, were dissolved in 3 ml. of glacial acetic acid, 43 mg. of semicarbazide hydrochloride (3.2 equivalents) and 40 mg. of anhydrous sodium acetate were added, and the solution was heated to 90° on a steam bath for 50 minutes under a stream of nitrogen. The bissemicarbazone was precipitated with water, filtered, washed with water, and dried. The ultraviolet spectrum revealed a strong absorption at 2700 Å, $\epsilon = 27,000$ (methanol), which is characteristic for an α,β -unsaturated semicarbazone. This semicarbazone was dissolved in 2 ml. of glacial acetic acid and 0.4 ml. of pyruvic acid, 350 mg. of anhydrous sodium acetate, and 0.5 ml. of water were added, and the solution was refluxed at 110° for 50 minutes. During this period, an additional 1 ml. of water was added. The solution was transferred with about 50 ml. of ether to a separatory funnel and washed with water, 5 per cent sodium carbonate, and water. The ether layer was dried over sodium sulfate, decanted, and evaporated. Progesterone crystallized from ether. The total residue, 39 mg., was sublimed at $90-100^\circ$ in a high vacuum. 36 mg. of material were obtained which yielded on recrystallization from ether-petroleum ether 30 mg. or 80 per cent of crystalline progesterone, m.p. $118-121^\circ$.

*17 β -Acetoxy-*d₂-11,12-etiocholan-3-one**—240 mg. of 17 β -acetoxy-*d₂-11,12-etiocholan-3 α -ol* were oxidized with 55 mg. of CrO_3 (2.2 equivalents) in glacial acetic acid at 10° overnight. The product was worked up in the usual manner and purified by chromatography over alumina. 17 β -Acetoxy-*d₂-11,12-etiocholan-3-one* was obtained as prisms, m.p. $149-151^\circ$, rearranging at 146° ; $[\alpha]_D = +31.5^\circ$ (CHCl_3) in a yield of 80 per cent.

$\text{C}_{21}(\text{H} + \text{D})_{20}\text{O}_2$ (334.09).	Calculated.	C 75.21, H + D 10.14
	Found.	" 75.49, " + " 10.05

*4-Bromo-17 β -acetoxy-*d₂-11,12-etiocholan-3-one**—5.5 ml. of a 0.48 M solution of bromine in acetic acid (2.10 equivalents) were added dropwise within 15 minutes to a chilled solution of 400 mg. of 17 β -acetoxy-*d₂-11,12-etiocholan-3-one* in glacial acetic acid, with the rate of addition determined by the decolorization of the bromine previously added. After evaporation to a few ml. at 40° under reduced pressure, ether was added and a crop of well developed octahedral prisms was obtained. After recrystallization from chloroform-ether, this product, 174 mg. or 35 per cent, and hereafter

called "first fraction," melted at 185–192°, $[\alpha]_D = +36^\circ$ (CHCl_3). The mother liquors afforded further crystalline bromo compound which was recrystallized from chloroform-ether. 150 mg. (30 per cent) of similar octahedral prisms were obtained (called hereafter "second fraction"), m.p. 175–180°, with no depression of melting point when admixed with the first fraction. The compound, 4-bromo-17 β -acetoxy- d_2 -11,12-etiocholan-3-one, was, however, more dextrorotatory, $[\alpha]_D = +46^\circ$ (CHCl_3), than the first fraction.

$\text{C}_{21}(\text{H} + \text{D})_{11}\text{BrO}_2$ (413.01). Calculated, Br 19.35; found, Br 19.35

The total yield of crystalline product was 65 per cent. The mother liquor, 160 mg., was debrominated with zinc as described for the similar fraction from bromo- d_2 -11,12-pregnandione and 80 mg. (20 per cent based on the starting material) of 17 β -acetoxy- d_2 -11,12-etiocholan-3-one were recovered.

d_2 -11,12-Testosterone Acetate—100 mg. of pure 4-bromo-17 β -acetoxy- d_2 -11,12-etiocholan-3-one (second fraction, $[\alpha]_D = +46^\circ$) were dehydrobrominated by the modified procedure already described. The crystalline residue was sublimed in a high vacuum at 90°. The yield was 73 mg. (90 per cent) after recrystallization from ether-petroleum ether; needles, m.p. 138–140°, $[\alpha]_D = +92^\circ$ (CHCl_3), $\epsilon_{2410} = 17,400$ (methanol). For isotopic analysis, the product was converted to testosterone; 5.77 atoms per cent excess deuterium (1.62 atoms of deuterium per mole) were found.

2,4-Dibromo-17 β -acetoxy- d_2 -11,12-etiocholan-3-one—100 mg. of the impure bromo compound (first fraction, $[\alpha]_D = +36^\circ$) were dehydrobrominated exactly as in the preceding experiment. From the residue on addition of ether, a bromo compound crystallized and after recrystallization, 20 mg. of square prisms, m.p. 187–192°, $[\alpha]_D = +12.5^\circ$, were obtained from chloroform-ether.

$\text{C}_{21}(\text{H} + \text{D})_{10}\text{O}_2\text{Br}_2$ (475.91). Calculated, Br 32.50; found, Br 31.19

The infra-red spectrum exhibited the same absorption band at 1758 cm^{-1} as that shown by 2,4-dibromo- d_2 -11,12-pregnane-3,20-dione, which demonstrated that the compound was a 2,4-dibromo 3-ketone. The ether-soluble fraction containing the d_2 -11,12-testosterone acetate was chromatographed on a column that contained 20 times its weight of alumina. 5 per cent to 50 per cent benzene in petroleum ether eluted 52 mg. of crude d_2 -11,12-testosterone acetate, or 60 per cent, which was sublimed at 90° in a high vacuum and recrystallized from ether-petroleum ether together with the product obtained from the second fraction.

It is a pleasure to acknowledge the cooperation of Dr. K. Dobriner of this Institute who determined and interpreted the infra-red spectra for us.

SUMMARY

1. d_2 -11,12-Progesterone and d_2 -11,12-testosterone were prepared from Δ^{11} -lithocholenic acid.

2. 17 β -Acetoxyetiocholan-3 α -ol was obtained from pregnanolone formate by oxidation with perbenzoic acid and removal of the formyl group on alumina.

3. Pregnane-3,20-dione and 17 β -acetoxyetiocholan-3-one were found to yield, in addition to the 4-monobromo derivative, 2,4-dibromo 3-ketones upon bromination with 1 mole of bromine.

4. With the purified 4-bromo-3-ketocholane compounds, dehydrobromination was accomplished readily and in high yield by a modification of the Mattox-Kendall procedure. Isolation of the dinitrophenylhydrazone was unnecessary and the omission of HBr in the cleavage with pyruvic acid was advantageous. An analogous procedure with semicarbazide gave similar results.

5. Under the reaction conditions used the 2,4-dibromo 3-ketones were recovered unaltered.

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THE METABOLIC ACTIVITY OF THE α -, β -, AND γ -HYDROGEN ATOMS OF L-LEUCINE AND THE α -HYDROGEN OF GLYCINE*

By DAVID B. SPRINSON AND D. RITTENBERG

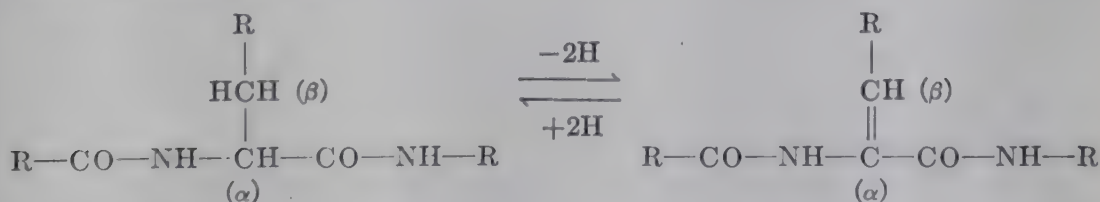
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An important conclusion derived from the application of the isotope technique to problems of intermediary metabolism is that many of the large molecules, usually associated with the structural constancy of the cell, are in fact labile. They are continuously entering metabolic pathways through a breakdown to and resynthesis from small molecules. The chemical reactions of the *intact* proteins are, however, of interest. Although it is known that the immune proteins can participate in certain specific reactions involving a definite portion of the molecular surface, hydrolytic reactions are the only examples of well defined biochemical reactions of protein molecules which are well understood.

The discovery of an enzyme capable of hydrolyzing dehydropeptides (2) and its wide-spread occurrence in animal tissues (3, 4) imply that the reversible dehydrogenation of peptides may be a metabolic reaction. This would conform with earlier suggestions by Dakin (5) and Bergmann *et al.* (6) concerning the possibility of enzymatic α, β -dehydrogenation of amino acids. The finding that, after heavy water has been administered to animals, electrically stimulated muscle incorporates more deuterium from the body fluids than does inactive muscle (7) could be explained by this type of reaction along a polypeptide chain.

It is clear from the following scheme for the dehydrogenation of a peptide that it would lead to a labilization of both α - and β -hydrogen atoms of amino acids.



In order to gain further insight into this problem leucine was synthesized, labeled with deuterium on the α -, β -, and γ -carbon atoms, as well as with

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N^{15} in the amino group, and the L isomer was fed to rats. Leucine was then isolated separately from the carcass and internal organs. By suitable degradative methods, the concentration of deuterium at each of the labeled positions was determined in the compounds fed and isolated. The D: N^{15} ratios for the α , β , and γ atoms are shown in Table I.

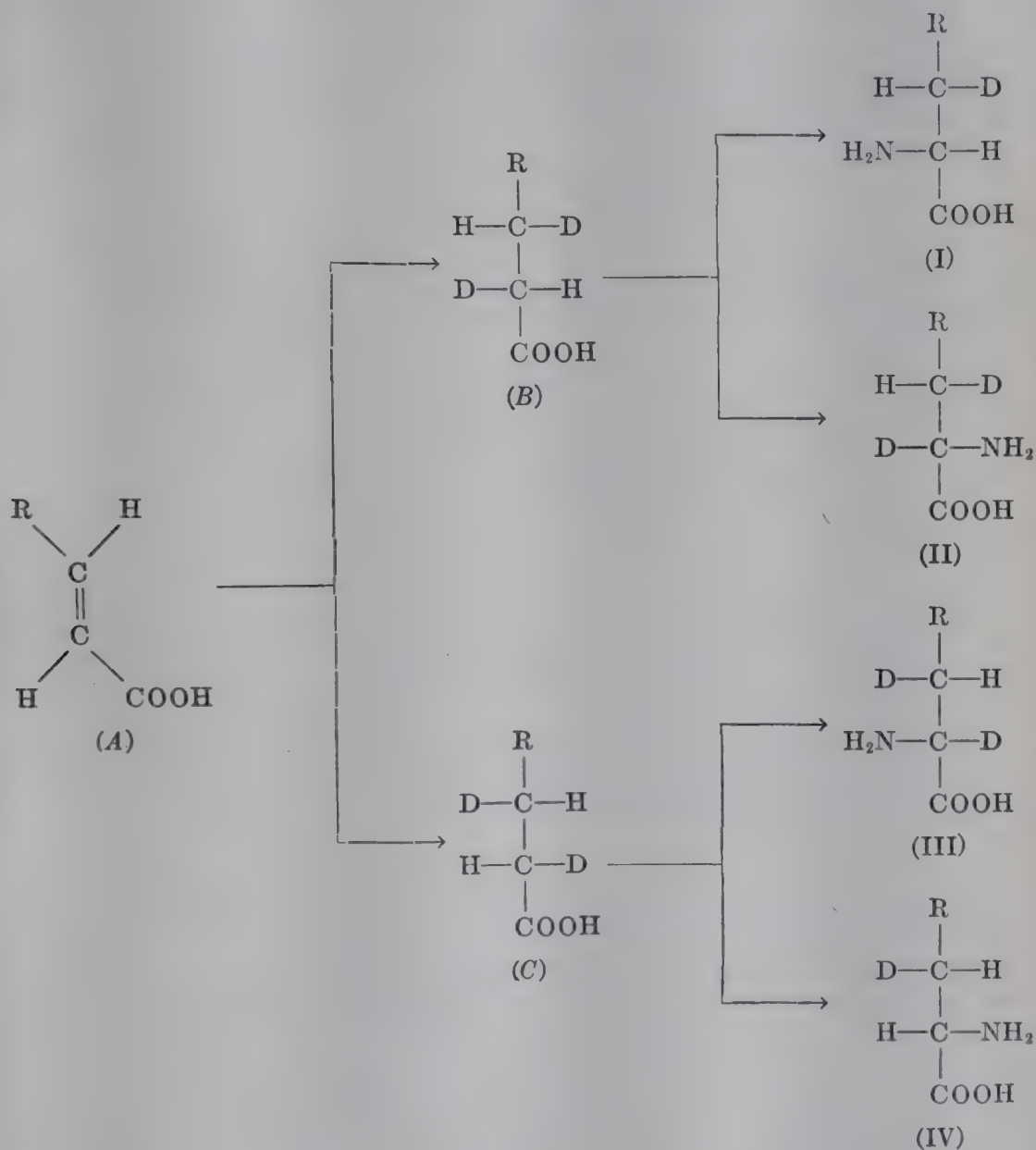
Reactions of β - and γ -Deuterium Atoms

If, as seems probable, the γ -hydrogen atom of leucine is metabolically stable, the change in the $D_\gamma:N^{15}$ ratio of the leucine after feeding it to an animal can be taken as a standard of reference to which any exchange reactions undergone by the α - and β -deuterium atoms can be compared. Actually the changes in the $D_\beta:N^{15}$ and $D_\gamma:N^{15}$ ratios, for leucine from both carcass and internal organs, show nearly the same dilution for the β - and the γ -deuterium atoms. Leucine, in protein or in peptide linkage, seems not to have participated in metabolic reactions during which the β -deuterium atom was subjected to extensive reversible α,β -dehydrogenation or other specific reactions. Of interest in this connection is the finding (8) that glycyldehydrophenylalanine is incapable of replacing phenylalanine in an *Escherichia coli* mutant requiring this amino acid, although glycylphenylalanine is readily utilized.

It is known from previous experiments with doubly labeled amino acids that the D: N^{15} ratio of lysine remains unchanged after administration to rats (9), a finding which implies the non-reversibility of nitrogen transfer in this amino acid. However, when L-leucine labeled with N^{15} and containing deuterium approximately evenly distributed over the entire carbon structure was fed to rats, the D: N^{15} ratio was almost twice as high in the isolated material as in the compound fed (10). In addition to dilution with dietary and tissue leucine, the isotopic leucine must have participated in chemical reactions leading to removal of nearly one-half of the N^{15} and its replacement by normal nitrogen. The increase in the $D_\beta:N^{15}$ and $D_\gamma:N^{15}$ ratios after feeding α,β,γ -deuterio- N^{15} -leucine is approximately the same, in the leucine from both carcass and internal organs, as that reported previously (10) for the over-all (random) D: N^{15} ratios. This is additional support for the view that the β - and γ -deuterium atoms accompany their carbon atoms during the reversible reactions involving the α -carbon atom.

It has been assumed in this discussion that both of the β -hydrogen atoms of the administered leucine are equally labeled with deuterium. If this is not so and only 1 of the 2 β -hydrogen atoms is labeled, it is conceivable that α,β -dehydrogenation could have proceeded without being detected, since the spatial specificity of the enzyme may be such that only 1 of the β -hydrogen atoms of the amino acid is involved in the dehy-

drogenation. It is possible to show that the L-leucine employed in this investigation contained deuterium in both β -hydrogen positions. If for example the isopropylacrylic acid (A) used in the synthesis were the trans form, two molecular species of deuterioisocaproic acid (B + C)



may be formed on addition of deuterium. This would correspond to the DL and LD forms derived by addition of bromine to *trans*-isopropylacrylic acid, or the formation of DL-tartaric acid from fumaric acid. Since both hydrogen atoms on the α -carbon atom of the deuterioisocaproic acid are equivalent, bromination proceeds at random, resulting in the loss of half

of the deuterium on the α -carbon atom of both species and the appearance of two pairs of isotopic isomers. Amination of the bromo compounds will yield four isotopic isomers of leucine (I, II, III, and IV). Compounds II and IV have the same configuration about the α -carbon atom and are L-leucine; I and III are D-leucine. Either of these optically active amino acids will contain deuterium in both β -hydrogen positions. The same considerations would apply if the starting isopropylacrylic acid were the cis form or any mixture of cis and trans.

Reactions of α -Deuterium Atom

An entirely different situation is presented by the reactions of the α -deuterium atom of carcass leucine. The ratio of $D_\alpha:N^{15}$ fell appreciably during interaction with the carcass proteins. It can be seen from Table

TABLE I
Metabolic Changes in Deuterium- N^{15} Ratios of α -, β -, and γ -Deuterium Atoms of L-Leucine

Ratio of D: N^{15} *	L-Leucine			Isolated-fed†	
	Fed	From carcass	From internal organs	Carcass	Internal organs
$D_\alpha:N^{15}$	1.46	0.38	1.53	0.26	1.05
$D_\beta:N^{15}$	3.72	8.05	5.53	2.17	1.49
$D_\gamma:N^{15}$	1.15	2.45	1.83	2.13	1.59

* The data from which these ratios have been calculated are in the text.

†
$$\frac{\text{Ratio (D:N}^{15}\text{) in leucine isolated}}{\text{Ratio (D:N}^{15}\text{) in leucine administered}}$$

I that the α -deuterium atom was replaced about 4 times as extensively as the N^{15} , and, since the $D_\beta:N^{15}$ ratio rose by a factor of 2, the replacement of the α -deuterium atom was 8 times as great as that of the β or γ atoms. The deuterium concentration on the α -carbon atom (0.22 atom per cent excess) is, nevertheless, about 10 times that of the body water (0.021 atom per cent excess). Results somewhat similar to those shown by the α -deuterium atom of carcass leucine were obtained when L- α -amino- γ -phenylbutyric acid labeled with N^{15} was fed to rats in conjunction with heavy water (11). Although the excreted acetyl-L-aminophenylbutyric acid lost only a small proportion of its N^{15} , 1 atom of deuterium entered the α position. When the body fluids of mice were enriched with heavy water most of the amino acids isolated from the tissue proteins contained stably bound deuterium (12). It is reasonable to assume that a part of this was located in the α position.

Additional evidence bearing on this question is summarized in Table II. Glycine, labeled with deuterium on the methylene carbon atom and N^{15} in the amino group, was injected or fed to rats in conjunction with benzoic acid. The change in the ratio of $D:N^{15}$ in the glycine excreted as hippuric acid shows that loss of α -hydrogen atoms can occur without concomitant loss of the α -nitrogen. This loss of α -hydrogen does not take place after hippuric acid is formed, for following the administration of α -deuteriohippuric acid the excreted hippuric acid was undiluted (see the experimental part). Specific reactions of the α -carbon atoms must be involved in the case of glycine, since α,β -dehydrogenation is, of course, impossible.

TABLE II
Lability of α -Hydrogen of Glycine During Hippuric Acid Formation

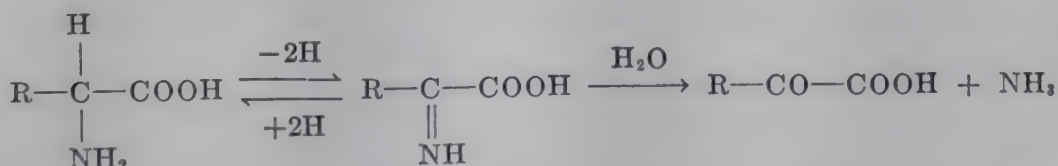
Experiment No.	Glycine administered*			Hippuric acid		Dilution	
	N^{15} concentration	D concentration	Dosage per 100 gm. rat	N^{15} concentration	D concentration	N^{15}	D†
	atom per cent excess	atom per cent excess	mM	atom per cent excess	atom per cent excess		
1	1.01	6.13	1.65	0.457	0.301	2.2	11
2	16.0	14.0	1.07	6.0‡	0.33‡	2.7	23
3		12.0	1.55		0.67		10

* The method of administration is described in the experimental part.

† Calculated on the basis of the D in the excreted glycine, which is the D of the hippuric acid times 9/5.

‡ Represents the average of the values obtained on 3 successive days.

An intermediate in the oxidative deamination of amino acids is assumed to be the imino acid.



If the rate of hydrogenation of the imino compound is of the same order of magnitude as its hydrolysis, then deuterium could leave or enter the α position without loss of nitrogen. There is as yet no direct evidence bearing on this point. Replacement of the α -deuterium atom has been observed (13-15) in experiments with purified transaminase preparations and α -deuterioamino acids. Apparently an exchange of deuterium took place even while the usual shift of amino groups catalyzed by this enzyme

was not detectable. A process of this type could account for the findings, reported here, in the intact animal.¹

EXPERIMENTAL

Synthesis of α,β,γ -Deuterioleucine Labeled with N^{15}

Isopropylacrylic acid was synthesized by the condensation of freshly distilled isobutyraldehyde² (6 moles) with malonic acid (5 moles) in the presence of 500 cc. of pyridine containing 0.5 per cent of piperidine (16, 17). After 4 days at room temperature the mixture was heated on the steam bath for 6 hours, diluted with 2 volumes of water, acidified to Congo red with sulfuric acid, and extracted with ether. Fractionation of the dried ether solution gave a 72 per cent yield of isopropylacrylic acid, b.p. 105–107° at 10 mm. Esterification with diazomethane in ether solution yielded the methyl ester, b.p. 44–45° at 8 mm., in 94 per cent yield.

The unsaturated ester (24 gm.) was reduced with deuterium gas without solvent in the presence of 2 gm. of $PtO_2 \cdot H_2O$. Uptake of gas ceased when 4.4 liters had been absorbed. After the removal of the catalyst the product was added dropwise, under a reflux, to a boiling solution of 0.35 mole of KOH in 20 cc. of water. The alkaline mixture was diluted with water, acidified to Congo red with sulfuric acid, and extracted with ether. Fractionation of the dried ether solution gave 20 gm. of deuterioisocaproic acid, b.p. 92° at 8 mm.

The isocaproic acid was brominated, esterified, and aminated with potassium phthalimide labeled with 23 atom per cent excess N^{15} , as previously reported from this laboratory (18). In the working up of the hydrolysate of ethyl phthalimidoisocaproate there is a tendency for leucine to crystallize with the phthalic acid after excess HCl has been removed *in vacuo*. It is then necessary to extract the phthalic acid residues with dilute HCl in order to bring all the amino acid into solution. The

¹ Although the α -deuterium atom of the internal organ leucine is more diluted than the β - and γ -deuterium atoms, it is not replaced more extensively than the N^{15} . The extraordinary lability of the α -hydrogen atom of leucine appears to be confined to the carcass proteins. This finding seems especially curious when it is recalled that both the acetylation of L- α -amino- γ -phenylbutyric acid and the formation of hippuric acid, which are reactions accompanied by α -hydrogen labilization, are thought to take place in the liver. The transaminase activity *in vitro* of the internal organs (heart, kidney, cortex, liver) is about the same as that of skeletal muscle (15); consequently the possible rôle of this enzyme in the lability of the α -hydrogen atom of amino acids does not readily explain the difference in lability of the α -deuterium atom between internal organ and carcass leucine.

² We are indebted to Dr. Roland Kapp of the National Oil Products Company for the isobutyraldehyde used in this investigation.

yield of leucine in this synthesis was 15 gm. Resolution of a 12.7 gm. portion through the formyl derivative with brucine (10) afforded 4.6 gm. of L-leucine. It contained 11.0 atom per cent excess D and 22.6 atom per cent excess N¹⁵.

$C_6H_{13}O_2N$ (132.8).³ Calculated, N 10.7;³ found, N 10.6³
 $[\alpha]_D^{25} = +14.2^\circ$ (3.60 per cent in 20 per cent HCl)

The specific rotation for pure L-leucine under our conditions being 14.8° (19), the labeled material was about 98 per cent optically pure.

Degradation of Leucine

Determination of Deuterium Concentration at α Position—The isotope concentration at the α -carbon atom was determined by degrading the deuterioleucine to isovaleric acid, NH_3 , and CO_2 (20). A suspension of washed silver oxide, prepared from 2 gm. of $AgNO_3$, in a solution of 200 mg. of leucine in 30 cc. of water was refluxed for 3 hours, cooled, acidified with 1 cc. of concentrated H_2SO_4 in the cold, and filtered. Filtrate and washings were distilled to about half the volume, and the distillate was brought to pH 7 to 8 with dilute NH_4OH and treated with $AgNO_3$. The silver isovalerate was washed with cold water, ethanol, and ether. Yield, 90 to 200 mg. The product from the synthetic deuterioleucine contained 12.2 atom per cent excess D.

$C_6H_9O_2Ag$. Calculated.³ C 28.6, H 4.9, Ag 51.3
 210.1³ Found. " 28.7, " 4.8, " 51.2

In a control reaction with normal leucine in heavy water (4.5 atom per cent excess D) the silver isovalerate contained negligible amounts of deuterium (0.015 ± 0.001 atom per cent excess D). The silver oxide degradation is, therefore, a reliable method for eliminating the α -deuterium atom without labilization of the β and γ atoms.

Determination of Deuterium Concentration at γ Position—The isotope concentration at the γ -carbon atom was determined by degrading the deuterioleucine with chloramine-T (21): $(CH_3)_2CHCH_2CHNH_2COOH \rightarrow [(CH_3)_2CHCH_2CHNCl_2COOH] \rightarrow (CH_3)_2CHCH_2CN + 2HCl + CO_2$. The active α -hydrogen atoms of nitriles are labile in the presence of alkali (22) and can be eliminated. Hydrolysis, following exchange, should yield isovaleric acid containing only the γ -deuterium atom of leucine. The completeness of the exchange can be determined by carrying out the same

³ Corrected for isotope content.

reaction on normal leucine in a medium of heavy water. The α -hydrogen atoms of the intermediate isovaleronitrile should equilibrate with the deuterium of the medium, resulting in isovaleric acid which contains nearly 2 atoms of deuterium. When this control reaction was carried out by the procedure given below it was found that the exchange was only 80 per cent complete. This was taken into account when the deuterium concentration of the γ position was calculated.

To 100 mg. of deuterioleucine dissolved (with warming) in 5 cc. of 1.7 M citrate buffer, pH 4.6, were added at room temperature 0.7 gm. of chloramine-T in 10 cc. of water. The mixture was warmed at 45° under a reflux with frequent shaking for 0.5 to 1 hour and diluted with 10 to 15 cc. of water, and about 20 cc. of liquid were distilled off. Droplets of isovaleronitrile were observed at the surface of the distillate. After addition of 0.5 gm. of Na_2CO_3 the solution was allowed to stand at room temperature for 2 days and for 6 hours longer following addition of 2 gm. of NaOH. The mixture was then heated under a reflux on a steam bath for 16 to 20 hours and about 5 cc. of water were distilled off and discarded. The cooled alkaline solution was acidified with 10 cc. of 10 N H_2SO_4 and about 20 cc. of water distilled off. Silver isovalerate was precipitated from the distillate as previously described. Yield 102 mg. It contained 4.8 atom per cent excess D.

The degradation of normal leucine in D_2O was carried out in a similar manner. L-Leucine (100 mg.), citric acid monohydrate (0.89 gm.), and sodium citrate dihydrate (1.76 gm.) were dissolved in 5 cc. of 99 per cent D_2O . Chloramine-T (0.7 gm.) in 10 cc. of D_2O was added and the reaction conducted as previously described. 5 to 10 cc. of D_2O were added and 12 to 17 cc. of liquid distilled off. The base-catalyzed exchange reaction was now carried out on the distillate as before, the nitrile hydrolyzed, 3 cc. of the solution distilled off and discarded, and another small portion distilled off and saved for the determination of the deuterium concentration of the medium. The silver isovalerate was isolated from the distillate obtained after acidification. Yield 60 to 80 mg. In a typical experiment the deuterium concentration of the reaction medium was 85 per cent, while that of the silver isovalerate was 15.7 per cent. The theoretical exchange for the 2 α -hydrogen atoms of isovaleronitrile is 2×0.85 . The observed exchange was 9×0.157 , or 83 per cent of theory.

Distribution of Deuterium in α , β , and γ Positions; on Reduction of Isopropylacrylic Acid—The deuterium concentrations at the α -, β -, and γ -carbon atoms can be calculated as shown in the following example for the synthetic deuterioleucine. It should be remembered that the number of atoms of deuterium in leucine and silver isovalerate is 13 and 9 times the D concentration, respectively.

$$D_{\alpha} = (13 \times 0.110) - (9 \times 0.122) = 0.33 \text{ atom}$$

$$0.2D_{\beta} + D_{\gamma} = 9 \times 0.048 = 0.43 \text{ atom}$$

$$D_{\beta} + D_{\gamma} = 9 \times 0.122 = 1.1 \text{ atoms}$$

$$0.8D_{\beta} = 1.1 - 0.43 = 0.67 \text{ atom}$$

$$D_{\beta} = 0.84 \text{ atom}$$

$$D_{\gamma} = 0.26 \text{ atom}$$

The results of the degradation show that in the reduction of isopropylacrylic acid appreciable amounts of deuterium had been introduced into the γ position. It is uncertain how this occurred. The isopropylacrylic acid could have contained the β, γ -unsaturated isomer or the latter might have been formed by a rearrangement of the double bond system in the presence of the platinum catalyst. In either case deuterium would be introduced into the γ position and a deficit would result on the α -carbon atom. It is possible that the reduction involved mainly the α, β -unsaturated acid and that deuterium was introduced in the γ position by exchange. Exchange reactions of the γ -hydrogen atom of isopropylacrylic acid would be in keeping with the known mobility (17, 23) of this conjugated double bond system in the presence of alkali: $(\text{CH}_3)_2\text{CH}-\text{CH}=\text{CH}-\text{COOH} \rightleftharpoons (\text{CH}_3)_2\text{C}=\text{CH}-\text{CH}_2-\text{COOH}$.

Leucine Feeding Experiments and Isolation Procedures

Feeding of α, β, γ -Deuterio- N^{15} -leucine—Four adult male rats having a combined weight of 940 gm. were kept on a diet of the following composition for 3 days: 84 per cent corn-starch, 4 per cent brewers' yeast, 4 per cent salt mixture (24), 6 per cent Wesson oil, and 2 per cent cod liver oil. This low protein diet was used in order to minimize dilution of isotopic material with normal leucine. The labeled amino acid was dissolved in an equivalent amount of NaHCO_3 solution, mixed into the diet with enough water to make a paste, and fed to the rats for the next 3 days. Altogether 3.4 gm. of α, β, γ -deuterio- N^{15} -L-leucine were consumed in 117 gm. of ration. During the 6 day period there was a loss in weight of 6.5 per cent. At the end of the 3rd feeding day the rats were killed and the bodies separated into internal organs (liver, washed intestine, spleen, kidneys, testes, heart, and serum proteins) and carcass (remainder without the skin). A sample of body water distilled from the pooled internal organs showed a deuterium concentration of 0.021 atom per cent excess.

Carcass Leucine—The combined minced carcass was extracted with 6 per cent trichloroacetic acid and the insoluble residue hydrolyzed with 1600 cc. of 20 per cent HCl . After removal of the acid *in vacuo*, the syrup was taken up in about 1.5 liters of water, treated with a large excess of Cu_2O (25), chilled, filtered, and the filtrate treated with H_2S and norit. The cleared solution was concentrated to about 300 cc. (accompanied by precipitation). On standing overnight in the refrigerator a copious mass

of tyrosine and leucine was obtained. This was filtered off and the filtrate adjusted to pH 5 to 6, concentrated to 300 cc., and allowed to stand in the cold for a few days. The precipitate ("leucine fraction") was collected.

The above mixture of leucine and tyrosine was recrystallized from water, giving 1.2 gm. of crude tyrosine; the filtrate was concentrated to a small volume and yielded 5.5 gm. of L-leucine 2-bromotoluene-5-sulfonate (26). An additional 1.5 gm. of this salt were obtained from the leucine fraction. The combined salts were recrystallized from 50 cc. of 0.1 N HCl and the resulting 5.6 gm. of material were decomposed with $\text{Ba}(\text{OH})_2$ (26). The barium-free filtrate was concentrated and the leucine precipitated with absolute ethanol from its solution in a small volume of hot water. Yield 1.4 gm. The mother liquor was taken to dryness and the residue, crystallized from a small volume of water, gave 0.32 gm. The main crop was recrystallized from water (about 25 cc.) and gave 0.43 gm.

$\text{C}_6\text{H}_{13}\text{O}_2\text{N}$. Calculated. C 55.0, H 10.0, N 10.7

131.2 Found. " 55.2, " 10.0, " 10.7, S 0.2

$[\alpha]_D^{25} = +15.0^\circ$ (3.51 per cent in 20 per cent HCl)

Degradation of Carcass Leucine—The methods described for the degradation of the synthetic leucine were also applied to the leucine isolated from carcass. This had 0.48 and 0.572 atom per cent excess D and N^{15} respectively. The silver isovalerate from the Ag_2O oxidation had 0.67, while that from the chloramine-T and exchange reactions had 0.26 atom per cent excess deuterium. By calculations such as those for the synthetic compound these values show that $D_\alpha = 0.0022$ atom, $D_\beta = 0.046$ atom, and $D_\gamma = 0.014$ atom.

Leucine from Internal Organs—By procedures similar to those described for the carcass hydrolysate 0.7 gm. of leucine 2-bromotoluene-5-sulfonate were obtained from the proteins of the internal organs. Recrystallization from 0.1 N HCl gave 590 mg.

$\text{C}_7\text{H}_7\text{O}_3\text{SBr} \cdot \text{C}_6\text{H}_{13}\text{O}_2\text{N} \cdot \text{H}_2\text{O}$. Calculated. C 39.0, H 5.5, N 3.5, H_2O 4.5
400.2 Found. " 39.1, " 5.4, " 3.5, " 4.4

In order to provide sufficient material for degradation 0.542 gm. of the anhydrous salt was dissolved in 50 cc. of warm water with 1.528 gm. of anhydrous normal L-leucine 2-bromotoluene-5-sulfonate, the salt decomposed as previously described, and 450 mg. of leucine isolated.

$\text{C}_6\text{H}_{13}\text{O}_2\text{N}$. Calculated. C 55.0, H 10.0, N 10.7

131.2 Found. " 55.0, " 9.7, " 10.8

Degradation of Leucine from Internal Organs—The leucine from internal organs, corrected for the dilution with normal leucine, had 2.46 and 3.60 atom per cent excess D and N¹⁵ respectively. The silver isovalerate from the Ag₂O degradation had 2.95, and that from chloramine-T and exchange reactions had 1.18 atom per cent excess D. These values indicate that $D_\alpha = 0.055$ atom, $D_\beta = 0.199$ atom, and $D_\gamma = 0.066$ atom.

Investigation of Biochemical Lability of α -Hydrogen of Glycine

Preparation of α -Deuterio-N¹⁵-Glycine—200 mg. of platinum oxide in 10 cc. of 99 per cent D₂O were reduced with hydrogen gas, 6 gm. of glycine and 1 cc. of concentrated HCl were added, and the flask, after being cooled with solid CO₂, was evacuated, sealed, and shaken in an oven at 130° for 21 days. After the removal of the platinum, glycine was isolated by the usual method. It had 28 atom per cent excess D.

Feeding of Deuterioglycine—In Experiment 1, Table II, a solution of 4 mM of α -deuterio-N¹⁵-glycine and 2.5 mM of sodium benzoate was injected intraperitoneally into each of five rats which had been fasted for 6 hours. Hippuric acid was isolated from the pooled urine. In Experiment 2, 3 mM of glycine and 3 mM of sodium benzoate were incorporated into 12 gm. of a 15 per cent casein diet (10) and fed daily to a rat for 3 days. Hippuric acid was isolated from each day's urine and analyzed for D and N¹⁵. An additional experiment (No. 3) was performed with glycine labeled only with deuterium and administered by injection. Each of three rats received 3 mM of glycine and 1.5 mM of sodium benzoate. Hippuric acid was isolated from the pooled urine. The data for all experiments are presented in Table II. In control experiments 735 mg. of α -deuteriohippuric acid, labeled with 6.4 atom per cent excess D, were injected into three rats as the sodium salt. The recovered hippurate had 6.1 atom per cent excess D. A sample of deuterioglycine (3.07 atom per cent excess D) which was allowed to stand for 24 hours at neutral pH and 38° showed no change in deuterium content. Exchange even in 20 per cent HCl at 96° is slow (2 per cent per day) (27).

SUMMARY

L-Leucine labeled with deuterium in the α , β , and γ positions and N¹⁵ in the amino group, and glycine labeled with deuterium on the methylene carbon atom and N¹⁵ in the amino group were prepared. Degradative procedures were worked out for the determination of the deuterium concentration at the α , β , and γ positions of leucine.

After administration of the leucine and glycine to rats leucine and hippuric acid were isolated from the tissue proteins and urine, respectively. Since the β - and γ -deuterium atoms of leucine had undergone similar

dilutions, as compared to the N^{15} , it appears that α,β -dehydrogenation of this amino acid had not occurred.

The α -deuterium atom of the carcass leucine and of the hippuric acid experienced greater dilutions than the N^{15} and some possible causes for this lability are discussed.

We are indebted to Mr. I. Sucher for isotope analyses, Messrs. B. Kanner and Albert Light for technical assistance, and Miss R. Rother for the microanalyses.

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THE CONJUGATED STEROIDS

I. A FRACTIONATION PROCEDURE FOR THE RAPID ISOLATION OF ESTRIOL MONOGLUCURONIDE FROM HUMAN PREGNANCY URINE*

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A series of investigations has been initiated in this laboratory for the development of procedures which will permit the separation of the various conjugated steroids in human urine. The distribution of conjugated estriol in human pregnancy urine was chosen as one means of following fractionation procedures, since this steroid can be readily assayed. The purification techniques involved in these studies are based on two early findings: (a) During the course of the preparation of sodium estriol monoglucuronide (4) by the addition of methanolic NaOH to a methanolic solution of estriol glucuronide concentrate, it was observed that such concentrates showed a strong buffering power between pH 4 and 6. This would tend to indicate that the glucuronide is converted to glucuronide in methanol in this pH range. (b) The presence of sodium chloride reduces the solubility of butanol and aqueous solutions in each other and might, therefore, be expected to sharpen the distribution of many organic substances between butanol and water. The importance of the presence of about 10 per cent water in alcohol or pyridine for the removal of estriol glucuronide from residue concentrates¹ (3) lends support to this thesis.

The importance of the effect of pH and of sodium chloride saturation on the distribution of conjugated estrogen between aqueous solutions and butanol has therefore been investigated. These studies have permitted the development of a procedure whereby it is possible to concentrate the estriol glucuronide in human pregnancy urine by relatively few and simple steps to a degree of purity permitting its ready crystallization.

Materials and Methods

Pregnancy urine collected from patients 2 to 4 weeks ante partum has been shown to be a rich source material for estriol glucuronide (5) and was, therefore, used in these studies.

* This work was aided by grants from the United States Public Health Service and the Graduate School of the University of Minnesota.

¹ Cohen, S. L., and Marrian, G. F., unpublished data (1935).

The estriol concentrates were prepared for assay by the method of Cohen and Marrian (2), and assayed by the technique of Stevenson and Marrian (8). Since no attempt was made to remove the weakly phenolic estrogens (estrone and estradiol) quantitatively, the assays for the estriol content of the various fractions were not strictly quantitative. However, an adequate degree of accuracy was attained by these simplified procedures.

DIAGRAM 1

Distribution of Conjugated Estriol between Butanol and H₂O at pH 4.0 and 6.0

LPU₂; 40 liters (Fraction 1)

Concentrated *in vacuo* to 5 liters;
acidified to Congo red; 6 times with 0.2 volume BuOH*

Aqueous residue

BuOH (Fraction 2)

Concentrated *in vacuo* to 200 cc.; adjusted to pH 4.0;
6 times with 0.2 volume H₂O

H₂O, pH 4.0 (Fraction 3)

Adjusted to pH 6.0; 6 times with 0.2 volume H₂O

H₂O, pH 6.0 (Fraction 4)

BuOH, pH 6.0 (Fraction 5)

* In this and in subsequent experiments, when the urine was not extracted shortly after its collection, freed steroids were removed by preliminary extraction with ether.

EXPERIMENTAL

Effect of pH on Distribution of Conjugated Estriol between Butanol and Aqueous Solutions—A butanol extract of pregnancy urine was adjusted to pH 4.0 and washed with water; after readjusting the pH to 6.0 the butanol was again washed with water. This fractionation scheme is shown in Diagram 1, while residue weights and estrogen assays obtained for aliquots of the various fractions are shown in Table I. It can be seen that, while the conjugated estriol cannot be removed from butanol by washing with water at pH 4.0, it is readily extracted by the water at pH 6.0. At pH 4.5, 5.0, and 5.5 only partial distributions between butanol and water were effected. It was further found, as was to be expected, that the conjugated estriol could also be removed from butanol by aqueous solutions of sodium bicarbonate, sodium carbonate, or sodium hydroxide.

Effect of NaCl on Distribution of Conjugated Estriol between Butanol and

Aqueous Solutions—A portion (0.4 volume) of the H₂O, pH 6.0, extract (Fraction 4, Diagram 1 and Table I) was saturated with NaCl and extracted eight times with 0.2 volumes of butanol each time. The butanol extract was adjusted to about pH 3.5 and freed of NaCl by washing several times with small volumes of water. Aliquot assays are shown in Table II, Fractions 1 and 2, and demonstrate the removal of conjugated estriol

TABLE I

Distribution of Conjugated Estriol in Urine between Butyl Alcohol and Water at pH 4.0 and 6.0

Fraction No.	Extract	Total estriol	Total residue	Estriol concentration
		mg.	gm.	per cent
1	Urine (batch LPU ₃).....	531	1265	0.04
2	BuOH extract of urine.....	536	197	0.27
3	H ₂ O, pH 4.0, extract of BuOH.....	36	89	0.04
4	" " 6.0, " " "	458	81	0.57
5	BuOH after H ₂ O washings.....	80*	10.4	0.77

* Further assays showed this to be primarily free estrogen.

TABLE II

Effect of NaCl on Distribution of Conjugated Estriol between Aqueous Solutions and Butanol

Fraction No.	Urine batch	Extract	Total estriol	Total residue	Estriol concentration
			mg.	gm.	per cent
1	LPU ₃	H ₂ O, pH 6.0, extract	184	32	0.57
2	"	BuOH extract of H ₂ O, pH 6.0, after saturation with NaCl	168	10.5	1.6
3	LPU ₄	BuOH extract of NaCl-saturated 1% Na ₂ CO ₃ concentrate	0.9	0.031	2.9
4	"	BuOH extract of NaCl-saturated 0.1 N NaOH concentrate	0.2	0.035	0.57

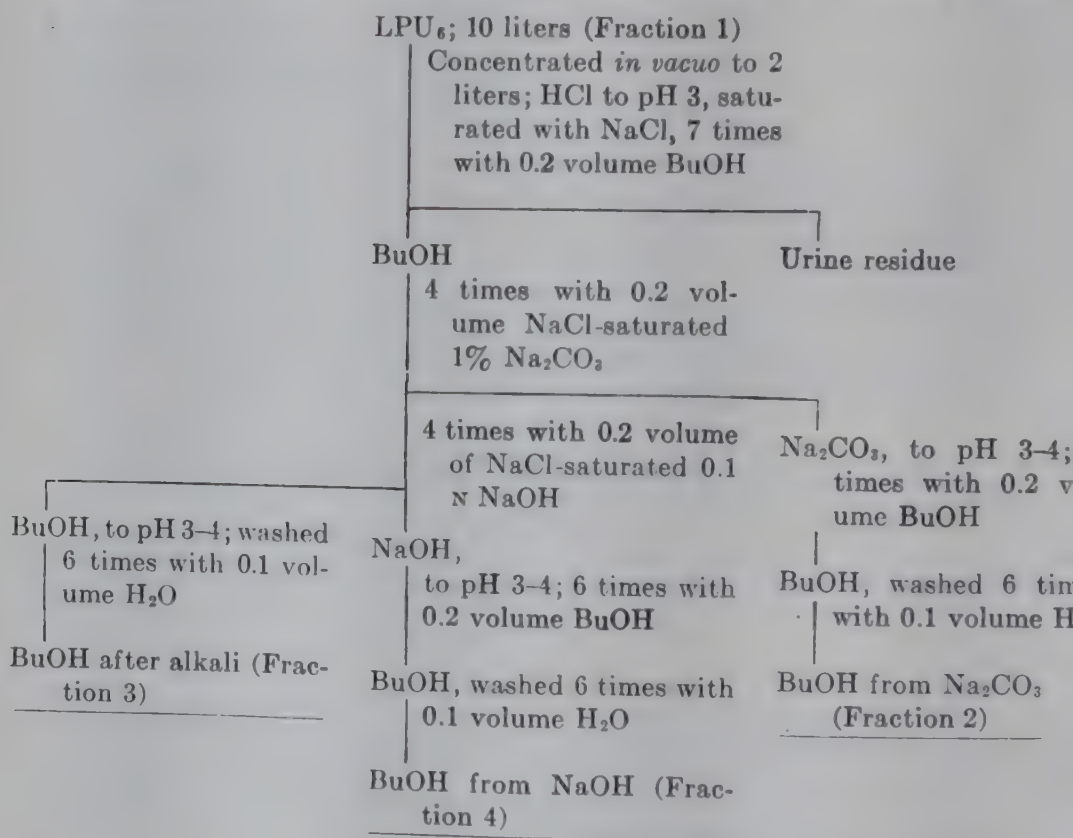
from H₂O at pH 6.0 by butanol in the presence of a NaCl-saturated solution.

It was further found that, while a Na₂CO₃ extract yielded its conjugated estriol to butanol after saturation with sodium chloride, NaOH extracts did not do so under similar circumstances. In a typical experiment two samples of 200 cc. of a butanolic extract of late pregnancy urine (batch LPU₄), each containing approximately 1 mg. of estriol in 70 mg.

of total residue, were extracted six times with 0.2 volume of aqueous per cent Na_2CO_3 and 0.1 N NaOH solutions respectively. The aqueous extracts were then saturated with NaCl and each extracted six times with 0.2 volume of butanol. The final butanol extracts were acidified to Congo red, freed of NaCl by washing several times with water, and assayed. The results are shown in Table II, Fractions 3 and 4. The data demonstrate that, while it is easy to remove the conjugated estriol

DIAGRAM 2

Preparation of Concentrates of Estriol Glucuronide from Pregnancy Urine



with butanol from a 1 per cent Na_2CO_3 concentrate after saturation with NaCl , it is very difficult to do so from an 0.1 N NaOH solution.

Preparation of Concentrates of Estriol Glucuronide from Pregnancy Urine. The effects of washing the original butanol extract of urine with NaCl -saturated Na_2CO_3 and NaOH solutions were investigated next. It was found, as expected, that washing of the butanol extract with NaCl -saturated Na_2CO_3 solution removed little estriol but considerable solid material including most of the pigmented material. Washing of the butanol extract with NaCl -saturated NaOH solutions removed the bulk of the conjugated estriol. A combination of these two procedures yields concen-

trates containing 4 to 10 per cent of colorimetrically assayable estriol (equivalent to 7 to 17 per cent of estriol glucuronide) from which sodium estriol glucuronide can readily be crystallized. A typical flow sheet for the concentration of conjugated estrogens by these procedures is shown for the urine in batch LPU₆ in Diagram 2 and the assay results in Table III.

The finding of an estrogen concentration of less than 5 per cent in the final "BuOH from NaOH" concentrate usually indicates the presence of considerable NaCl, which can be removed from the residue by washing with cold water. Thus, for the concentrate from batch LPU₆ (Fraction 4, Table III), washing five times with 10 cc. volumes of water at 2–5° left a residue of 1.1 gm., containing 93 mg. of estriol (*i.e.*, 8.5 per cent estriol concentration).

TABLE III

Distribution of Conjugated Estriol in NaCl-Alkali Concentration Process for Urine Batch LPU₆

Fraction No.	Extract	Total estriol	Total residue	Estriol concentration
		mg.	gm.	per cent
1	Urine.....	130		
2	BuOH from Na ₂ CO ₃	8.0	4.2	0.19
3	“ after alkali.....	9.8	2.6	0.38
4	“ from NaOH.....	101	2.5	4.0

Isolation of Pure Estriol Glucuronide—It has been possible to separate crystalline sodium estriol glucuronide from all five separate batches of pregnancy urine prepared by the procedures described above. The crystallization of the sodium salt has been effected by the technique previously reported (4). Thus, for example, the LPU₆ fraction (containing, as described above, 93 mg. of estriol in 1.1 gm. of residue) was dissolved in 50 cc. of absolute methanol, the pH brought to approximately 7.5 by the addition of methanolic NaOH, and the solution concentrated to about 5 cc. on a steam bath under a stream of nitrogen. After cooling, 106 mg. of a light brown crystalline precipitate melting with decomposition at 252–256° (uncorrected) were obtained.

Attempts to prepare crystalline estriol glucuronide have as yet (3, 4) not been successful. A purified though amorphous sample of estriol glucuronide was prepared as follows: 25 mg. of sodium estriol glucuronide were dissolved in 10 cc. of H₂O and 0.1 N HCl was carefully added at room temperature until precipitation was complete. Solution of the estriol glucuronide was effected by heating to boiling and a small amount of insoluble

brown residue was removed by filtering the hot solution. On cooling, jelly-like insoluble material formed. This was clarified by the addition of ethyl alcohol and the whole evaporated to dryness *in vacuo*. The residue was suspended in a small amount of cold H_2O , filtered, and the precipitate washed first with cold H_2O and then with cold ether. In this way 8 mg. of white material which melted with decomposition at $199-201^\circ$ (uncorrected) were obtained.

Identification of Isolated Estriol Glucuronide

Elementary Analyses. (a) *Sodium Estriol Monoglucuronide*—The crude material was recrystallized twice from methanol to yield crystals which melted with decomposition at $282-283^\circ$ (uncorrected). For analysis the

TABLE IV

Estrogen and Glucuronic Acid Content of Sodium Estriol Monoglucuronide (NaEG)

NaEG taken	Estriol found	Glucuronic acid found	Content in NaEG of	
			Estriol	Glucuronic acid
γ	γ	γ	<i>per cent</i>	<i>per cent</i>
30	16	11.5	53.3	38.2
60	33.5	24.0	55.8	40.0
60	34.5		57.8	
Average.....			55.5	39.1

$C_{24}H_{31}O_9Na$. Calculated, estriol 59.2, glucuronic acid, 39.7.

sample was dried at 120° for 10 hours at 0.1 mm. of Hg pressure. (Sodium was analyzed with the flame photometer.²)

$C_{24}H_{31}O_9Na$. Calculated. C 59.26, H 6.45, Na 4.73
Found. " 59.41, " 6.76, " 4.56

(b) *Estriol Monoglucuronide*—Estriol monoglucuronide was dried for analysis at 100° for 10 hours at 0.05 mm. of Hg pressure.

$C_{24}H_{31}O_9$. Calculated. C 62.03, H 6.95
Found. " 61.92, " 7.46

Estriol and Glucuronic Acid Content—The colorimetric method of Stevenson and Marrian (8) for estrogen was applied to aliquots of purified sodium estriol monoglucuronide. The glucuronic acid content was estimated by the method of Maughan, Evelyn, and Brown (7) (see Table IV).

² I wish to thank Miss E. Freier of the Laboratory Service of the University of Minnesota Hospitals for carrying out the sodium determinations.

DISCUSSION

The first preparations of estriol monoglucuronide from pregnancy urine (3, 4) involved preliminary concentration procedures with butanol, pyridine, quinoline, and alkali; the distributions between quinoline and alkali gave particularly troublesome emulsions. In the concentration procedures described in this paper, control of pH and saturation with sodium chloride alone in a few distributions of the estriol glucuronide between butanol and aqueous solutions permit the preparation of crystallizable concentrates of the estrogen. Furthermore, the presence of NaCl to a saturation level considerably reduces formation of emulsions.

The failure of Na_2CO_3 and the ability of 0.1 N NaOH to remove estriol glucuronide from butanol in the presence of NaCl saturation probably depend on the formation, in the latter case but not in the former, of a sodium salt on the free phenol group. Confirmation is lent to this hypothesis by an examination of the titration curves of estriol monoglucuronide and its comparison with that of molecularly equivalent amounts of related substances (Fig. 1). All solutions (in a 5 cc. volume) were adjusted to pH 2.0 to 2.5 by the addition of dilute HCl and electrometrically titrated with 0.2 N NaOH. It can be seen that estriol glucuronide in water (Curve 5, Fig. 1) shows one buffering range at pH above 9.0, corresponding to the buffering range of phenol (Curve 2, Fig. 1), and another buffering range below pH 4.5, corresponding with that of glucuronic acid (Curve 3, Fig. 1). These buffering areas are somewhat more acid than might be expected from the fractionation procedures described above. This is primarily due, however, to two causes: (a) the fact that the washings of the acid butanol extracts with 1 per cent Na_2CO_3 solutions under the conditions of the experiment result in aqueous extracts whose pH values do not exceed 9, and (b) the decreased dissociation of acids in alcohol solutions as compared to aqueous solutions (6). Thus, for example, estriol glucuronide in methanolic solution showed a pK at about 4.5 when titrated with methanolic NaOH (Curve 6, Fig. 1) as compared to a pK of about 3.0 in aqueous solution (Curve 5, Fig. 1). It thus seems highly probable that during the washing of the butanol extracts with NaCl-saturated 1 per cent Na_2CO_3 only the glucuronic acid portion of the estriol glucuronide molecule forms a sodium salt, whereas in the presence of a corresponding 0.1 N NaOH solution the acid groups of both the glucuronic acid and the phenol are present as sodium salts. In the latter instance the substance would tend to be even more soluble in water than in the former case and, therefore, washes out in the NaCl-saturated aqueous phase.

Saturation of the solvents with NaCl has the important property of reducing the solubility of butanol in water, thereby reducing the solubility of sodium estriol glucuronide in the water. Thus, while sodium estriol monoglucuronide is completely removed from butanol by water, if the

butanol content of the water is reduced by saturation with sodium chloride, the sodium estriol monoglucuronide becomes preferentially soluble in the butanol.

It is possible that the careful control of pH and the use of sodium chloride may help in the concentration of other conjugated steroids. This suggestion is supported by the finding that some of the conjugated 17-ketosteroids

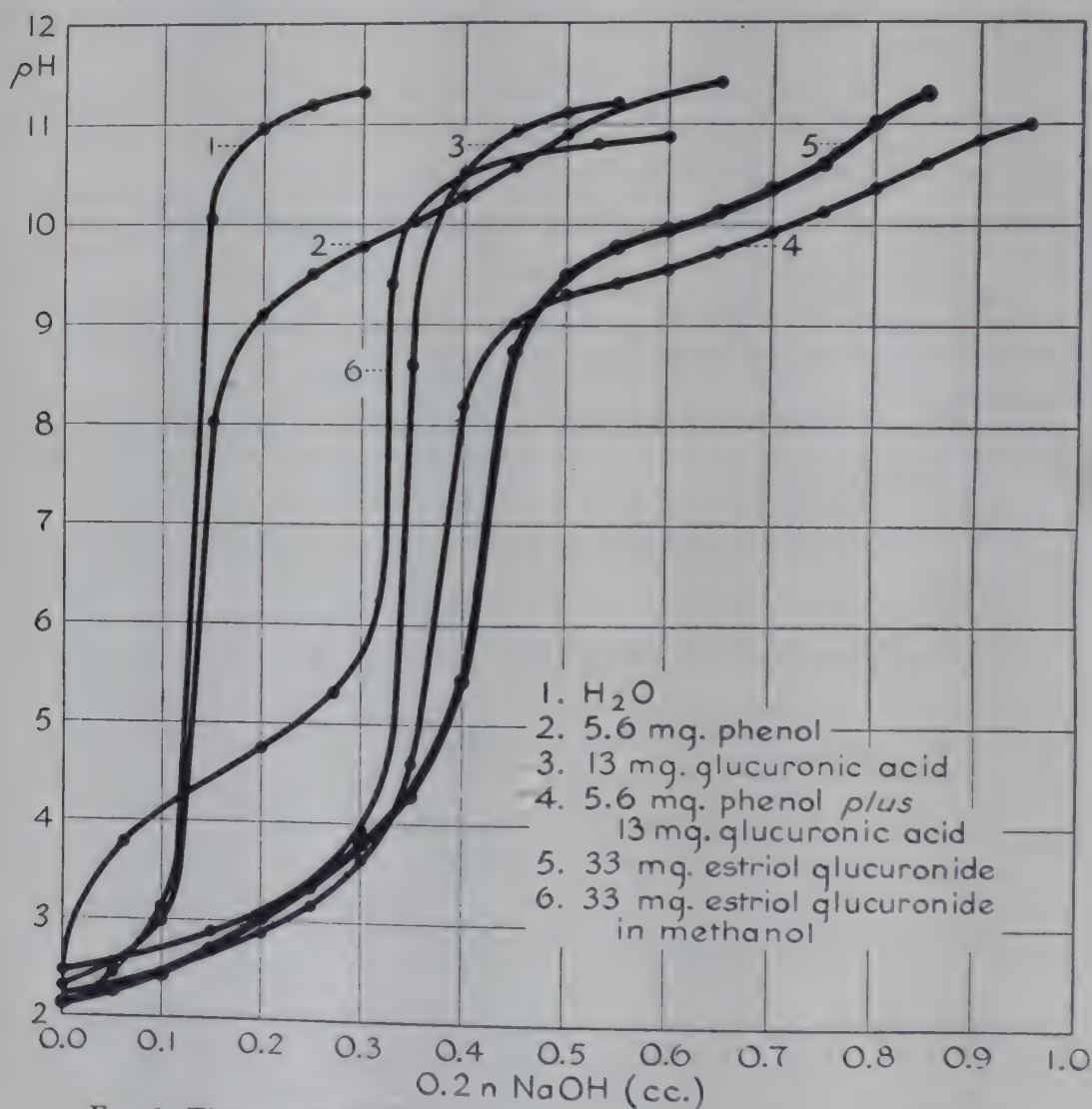


FIG. 1. Titration curves for estriol glucuronide and related substances

are not removed from urine by extraction with butanol at pH 7 but are removed at pH 3 or less (1).

SUMMARY

1. The influence of pH and of NaCl saturation on the distribution of estriol glucuronide of human pregnancy urine between butanol and aqueous solutions has been investigated.

2. As a result of these studies, a simple method for concentrating the estriol glucuronide has been devised by extracting an NaCl-saturated 1 per cent Na_2CO_3 -washed butanol extract of urine with NaCl-saturated 0.1 N NaOH.

3. Estriol glucuronide in 7 to 17 per cent concentration is thus obtained and can readily be crystallized as the sodium salt from methanol.

4. These concentration procedures depend in large part upon (a) the preferential solubility of sodium estriol monoglucuronidate in butanol when the butanol content of aqueous solutions is reduced by NaCl saturation, and (b) the formation of a monosodium salt of estriol glucuronide at pH 5 to 9 and of the disodium salt at pH values greater than 10.5.

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ON THE SYNTHESIS AND SOME APPLICATIONS OF SERINE- β -C¹⁴

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One of the serious difficulties encountered in tracer studies of protein synthesis *in vitro* is that of isolating a specific amino acid in a pure state from a protein hydrolysate to determine its activity. Udenfriend *et al.* (1) have shown that carrier recrystallization to constant activity, and even the synthesis of a derivative and its recrystallization, may not rid an amino acid of a radioactive impurity.

In a few cases radioactive determination of specific amino acids has been carried out with success. Thus, with S³⁵ as a label, methionine sulfur may be separated from cystine sulfur and determined as benzidine sulfate. Serine labeled in the β -carbon with C¹⁴ was thought to offer a useful tool for studies in which the amount of labeling due to serine only might be determined by submitting the protein hydrolysate to periodic acid oxidation according to the method of Nicolet and Shinn (2), and by determining the formaldehyde formed from the β -carbon as a derivative. With this end in view, experiments were initiated to synthesize serine labeled in the β -carbon with C¹⁴.

Serine has been synthesized with N¹⁵ (3) and doubly labeled with N¹⁵ and C¹³ in the carboxyl group (4). Both investigators used the formylation of labeled ethyl hippurate followed by a reduction and hydrolysis. The rather low over-all yield obtained by this method (5, 6) makes its use for the synthesis of β -labeled serine undesirable. For the serine used in the present experiments a modification of the King method (7) was used. Formaldehyde¹ labeled with C¹⁴ was condensed with acetamidomalonic ester and the condensation product was hydrolyzed stepwise to yield DL-serine- β -C¹⁴ in 67 per cent over-all yield, according to the accompanying scheme.

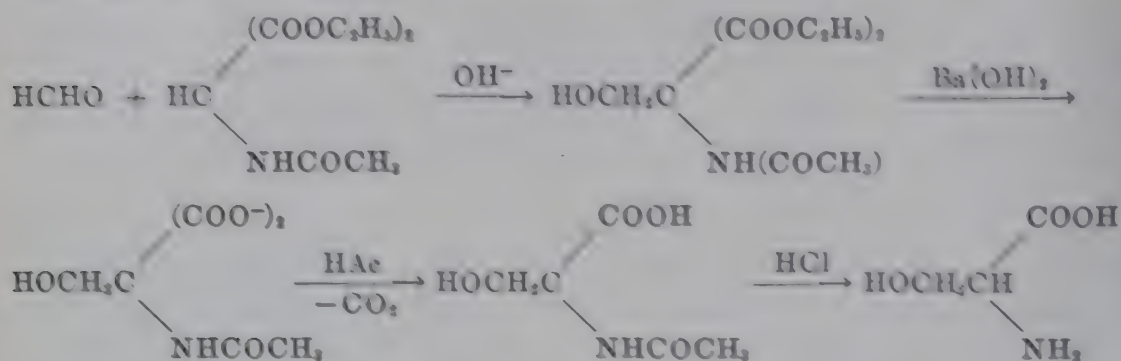
For experiments of the type projected, it also became necessary to develop a method which would allow the determination of the formaldehyde generated from the β -carbon of serine both with respect to amount and radioactivity.

Precipitation of the formaldehyde as the dimedon derivative provided

¹ The formaldehyde used was purchased from the United States Atomic Energy Commission, Oak Ridge.

the basis for a satisfactory method. The radioactivity in the derivative may readily be determined by the usual methods and its quantity by titration with a standard base.

Since uptake and distribution studies with β -labeled serine have not previously been carried out, it seemed of interest to study the distribution of the activity when the labeled serine is given to intact rats. Tracer doses were therefore injected into four female rats and the distribution of radioactivity of several tissues was determined both as protein and as serine by the method just indicated.



EXPERIMENTAL

Synthesis of DL-Serine- β -C¹⁴—20 ml. of 0.4 per cent solution of formaldehyde¹ containing 2.9 mm of formaldehyde and 6.27 mc. of C¹⁴ were added to 3.1 mm of ethyl acetamidomalonate and 2 drops of saturated Ba(OH)₂ in a 100 ml. round bottom flask. The flask was stoppered and allowed to stand at room temperature for 24 hours. The solution was then concentrated to 4 ml. under reduced pressure, the vapors being led through a series of scrubbers containing concentrated NH₄OH.² After standing 30 hours, 1.1 gm. of finely ground Ba(OH)₂·8H₂O (3.5 mm) were added, followed 12 hours later by 1 ml. of glacial acetic acid and 15 ml. of H₂O. Then the mixture was refluxed for 1 hour, the open end of the reflux condenser being connected to an NH₄OH trap. The clear yellow solution was evaporated to dryness over a boiling water bath under reduced pressure. The residue was dissolved with 8 ml. of hot water and refluxed with 20 ml. of 8 N HCl and 0.5 ml. of 10 N H₂SO₄ for 1 hour. The mixture was evaporated to dryness over a hot water bath at reduced pressure. Water was added and the last step was repeated. The contents of the flask were then transferred to a 100 ml. tube and all the Ba present was removed by addition of 1 N H₂SO₄ and centrifugation. The supernatant

¹ The function of the scrubbers is to catch the formaldehyde evolved. Pilot runs with dimedon in the scrubbers indicated that 10 to 20 per cent of the formaldehyde was split off during various steps of the reaction.

liquid was filtered onto a Duolite A-2 column, 2 cm. in diameter and 10 cm. long, the BaSO_4 was washed several times with hot water, and the washings were also passed through the column. 250 ml. of solution were collected and evaporated to dryness under reduced pressure. The crude serine was dissolved in hot H_2O , decolorized with norit A, and filtered. The solution was again taken to dryness. The white product was dissolved with the aid of 6 ml. of hot water and precipitated by the addition of 15 ml. of ethanol. After standing overnight, the crystals were filtered, washed with a mixture of 2 ml. of H_2O and 5 ml. of ethanol, and partially dried with suction. After recrystallizing once more, the product was dried over P_2O_5 . Yield, 160 mg. (58 per cent based upon formaldehyde).

170 mg. of recrystallized commercial DL-serine were added to the mother liquors and washings from the above product. After decolorizing and crystallizing twice, 161 mg. of DL-serine- $\beta\text{-C}^{14}$ were obtained with a radioactivity equivalent to 9 per cent of the original formaldehyde used.

Assay of DL-Serine- $\beta\text{-C}^{14}$ —Small portions of the product were diluted with inert serine and the radioactivity was determined both by sedimenting crystalline serine on aluminum disks and counting, and by treating the serine with periodic acid and trapping the β -carbon as the dimedon derivative of formaldehyde and determining its radioactivity. Assay as serine, 3.88×10^6 c.p.m. per mg. of serine; assay as MBM, 4.08×10^6 c.p.m. per mg. of serine.

Elementary analysis of serine prepared by the same method gave the following results:

Calculated.	C 34.28,	H 6.71,	N 13.33
Found.	" 33.75,	" 6.75,	" 12.68
	" 33.67,	" 6.74,	" 12.54

Determination of Formaldehyde As Methylenebismethane (MBM)—A pure sample of MBM was prepared according to Vorländer (8) (m.p. 191.0–191.5° (corrected); literature, 191.4° (9)). From this, a standard alcoholic solution containing 5 μM per ml. was prepared. Aliquots of this solution, in a total volume of 6.5 ml. of diluted alcohol, were titrated under various conditions with standard 0.01 N NaOH. Blanks were also titrated under similar conditions. Both the samples and the blanks contained a piece of Whatman No. 1 filter paper (diameter 4.25 cm.) to duplicate conditions under which the titration would be performed practically.

Results of these experiments showed that it is best to carry out the titration in a 10:3 alcohol-water mixture and in the absence of CO_2 . Moreover, it was found that if phenolphthalein is used as indicator, according to the method of Vorländer (10), the results obtained are invariably too high. The end-point of this indicator in the alcohol-water mixture is too

far on the alkaline side, so that the end-point is not sharp (11). If phenol red is used, correct results are obtained and the size of the blank is also reduced. The validity of the method for the range 5 to 25 μ M is shown by the data in Table I.

Radioactivity Determination—The radioactivity of the MBM and other substances was measured with a Tracerlab autoscaler, with a thin mica end window tube.³ Where necessary, corrections for self-absorption were applied.

Methods for Animal Experiments—Four adult female rats raised on laboratory rations were injected with a single tracer dose of serine labeled in the β -carbon with C¹⁴. Rats 1 and 2 received 0.5 mg. of a racemic DL-serine preparation containing 6×10^6 c.p.m. per mg. Rats 3 and 4 re-

TABLE I
Determination of Methylenebismethone by Titration

Methylenebismethone	Titration value*	
	In absence of CO ₂	In air
μ M	ml. 0.01 N NaOH	ml. 0.01 N NaOH
5	0.490 $\pm$ 0.009 (10)	0.502 $\pm$ 0.027 (7)
15	1.496 $\pm$ 0.007 (9)	1.476 $\pm$ 0.012 (7)
25	2.484 $\pm$ 0.012 (7)	2.530 $\pm$ 0.030 (7)
Blank	0.034 $\pm$ 0.002 (5)	0.063 $\pm$ 0.016 (5)
" †	0.105 $\pm$ 0.010 (5)	0.157 $\pm$ 0.070 (5)

* The value given is the mean $\pm$ standard deviation obtained from the number of determinations given in parentheses. The blank has been subtracted.

† Phenolphthalein was used as the indicator. In all other experiments phenol red was used.

ceived a mixture of 0.25 mg. of L- and 0.035 mg. of D-serine, in which the L-serine contained 5.40×10^6 c.p.m. per mg. and the D-serine 3.86×10^6 c.p.m. per mg.

Each rat was allowed food and water up to the time of injection, after which it was placed in a metabolism cage and allowed water for the duration of the experiment. Urine was collected under toluene, and CO₂ was collected in 2 N CO₂-free NaOH. The CO₂ was precipitated as BaCO₃, and washed four times with water and four times with acetone. 50 to 100 mg. samples were sedimented onto aluminum disks for counting, the self-absorption data of Yankwich and coworkers being used (12). Aliquots of the NaOH were titrated with standard acid with phenolphthalein and

³ We are indebted to the staff of the Crocker Laboratory, University of California, for generously supplying the tubes used in these experiments.

methlyl orange as indicators to determine total CO_2 . The activity of the whole urine was measured by drying suitable aliquots on aluminum disks and counting. The results as per cent of dose excreted are given in Table II.

After 6 hours the animals were sacrificed by ether anesthesia and heart puncture, and the livers, spleens, and kidneys as well as portions of muscle from the hind legs were removed, chilled, weighed, and homogenized in 10 per cent trichloroacetic acid, except that half of each liver was put aside. The protein precipitates were centrifuged and were then resuspended in 5 per cent trichloroacetic acid, and 100 mg. of inert DL-serine were added per gm. of original wet tissue, and the mixture was allowed to stand overnight in the ice box. The proteins were washed twice more with 5 per cent trichloroacetic acid. Nucleic acids were removed by heating the last

TABLE II

Excretion of Radioactive Carbon Following Injection of Serine- β - C^{14} into Rats

Per cent of total dose excreted in 6 hours as respiratory carbon dioxide or in the urine.

Rat No.	Weight	Per cent total C^{14} in	
		Urine	Respiratory CO_2
	gm.		
1*	233	4.3	45.8
2	232	2.5	45.9
3	250	3.6	44.9
4	238	2.7	35.1

* Rats 1 and 2 were given a racemic mixture of serine, whereas Rats 3 and 4 received a mixture containing only a trace of D-serine (see the text).

trichloroacetic acid-protein mixture to 91° for 15 minutes (13). Lipides were removed by washing once with hot absolute ethanol, twice with hot ethanol ether (3:1), and once with ether. Suitable portions of this protein preparation were sedimented onto aluminum disks and counted, the self-absorption corrections obtained with radioactive glycine being applied.⁴ The results are given in Table III.

Hydrolysis of Proteins and Determination of Serine Activity—The protein preparations were hydrolyzed by refluxing 20 hours with 8 N HCl. The major part of the HCl was removed by evaporating to dryness on the steam bath. The residues were dissolved in water and aliquots were treated with periodic acid by the method of Nicolet and Shinn (2), as modified by Rees

⁴ Goldsworthy, P. D., Greenberg, D. M., Peterson, E. A., Siekevitz, P., and Winnick, T., unpublished data.

(14). After aerating to remove the acetaldehyde formed by the oxidation of threonine, the mixture was acidified with sulfuric acid and steam-distilled into 15 ml. of a saturated dimedon solution. When the volume in the receiving flask was 100 ml., the distillation was stopped and the mixture was allowed to stand at room temperature for at least 20 hours. The MBM precipitate was then filtered, washed with three 10 ml. portions of hot water, and allowed to dry on the filter. The MBM was washed through the filter into a test-tube with hot acetone. The acetone solution

TABLE III

Uptake of Radioactive Carbon of Tissue Proteins of Rats Following Injection of Serine- β -C¹⁴

Per cent of total C¹⁴ taken up per gm. of tissue protein.

Rat No.	Muscle	Spleen	Liver	Kidney	Plasma
1	0.17	0.90	1.23	1.73	
2	0.19	0.90	1.23	1.73	1.77
3	0.19	1.27	1.43	1.97	2.08
4	0.20	1.62	1.47	2.55	2.44

TABLE IV

Uptake of Radioactive Serine by Tissue Proteins of Rats Following Injection of This Amino Acid

Per cent of total C¹⁴ taken up per mm of tissue serine $\times 10^3$.

Rat No.	Muscle	Spleen	Liver	Kidney
1	3.6	27.1	19.0	14.6
2	3.0	33.8	14.1	18.1
3	2.5		23.4	29.0
4	3.5	38.0	26.9	25.8

was evaporated to dryness on a water bath; the residue was redissolved with a minimal amount of acetone and reprecipitated by adding 10 ml. of water. After standing overnight, the precipitate was filtered onto Whatman No. 1 filter paper 4.25 cm. in diameter with the Tarver type filter (15). The radioactivity was determined as previously indicated. Self-absorption corrections were applied for C—N—H compounds on paper (16) and the amount of MBM was determined by titration, as already described. The results are given in Table IV.

The remaining half of each liver was pulverized with anhydrous Na₂SO₄ and was extracted for 4 days with chloroform in the Soxhlet apparatus.

The brains of Rats 3 and 4 were combined and similarly treated. The chloroform extracts were processed for the determination of ethanolamine and serine, according to the method of Artom (17), with the difference that the formaldehyde was steam-distilled into dimedon and determined both as to amount and radioactivity by the procedure already given.

To test whether radioactive serine could be separated adequately from ethanolamine by this method, 5 mg. of radioactive serine (with about 1100 c.p.m. per mg.) were mixed with 2 mg. of ethanolamine and separated by the Artom procedure. After oxidation with periodic acid, the formaldehyde from the serine and ethanolamine fractions was steam-distilled into dimedon and the MBM was processed and assayed by the usual procedure.

RESULTS AND DISCUSSION

From Table II it is seen that from 35 to 45 per cent of the administered C^{14} can be accounted for in the respiratory carbon dioxide 6 hours after injection. The low level of activity in the urine (2.5 to 4.3 per cent of the total C^{14}) agrees with results found when glycine is administered (18). Thus, there is no apparent difference in the metabolism of the two serine mixtures, judging from the results on the excreta, although there appears to be a difference in the uptake by the proteins.

The pattern of distribution of radioactivity in the several tissues (Tables III, IV) is the same as that found for methionine (19), with the highest level in the plasma proteins and kidney, liver, spleen, and muscle following in descending order. Rats 3 and 4, which received only 0.035 mg. of D-serine in the injected dose, have a slight but definitely higher level of uptake of activity into the protein. Table IV shows that the same is true for the β -carbon of serine; the average level of labeling is greater in Rats 3 and 4. This is true for all the tissues studied except muscle. The nephrotoxic action of D-serine in the rat is well known (20, 21), although the mechanism of action is not clear. The present tracer experiments indicate the possibility that D-serine inhibits the uptake of L-serine into the protein. However, it is also possible that in the animals given the smaller amount of D-serine the per cent conversion of D to the L form is greater, and that the specific activity of the L isomer is increased to a greater extent. This could also lead to the same results. Whether this action is related to the nephrotoxic effect must await further investigations.

If the average ratios between the figures of Tables III and IV are compared, it is immediately seen that this is not a constant but varies from tissue to tissue. Consequently the data of Table III, if taken alone, give incorrect values for the relative rates of incorporation of serine by the different tissues. The difference must be due to different rates of metab-

olism of serine by the different tissues, with consequent different rates of formation and incorporation of the label as other amino acids.

The results of the lipide fractionation indicate that there is activity in the ethanolamine of liver but not of brain 6 hours after injection (Table V). The figures for serine are lower than those for ethanolamine. This is believed to be due to an incomplete extraction of non-serine materials which can react with periodic acid to yield formaldehyde.

TABLE V
Radioactivities of Serine and Ethanolamine Fractions Isolated from Lipides of Rats Injected with Serine- β -C¹⁴

Tissue	Serine fraction		Ethanolamine fraction	
	c.p.m. per mg.	per cent C ¹⁴ per mm $\times 10^3$	c.p.m. per mg.	per cent C ¹⁴ per mm $\times 10^3$
Liver, Rat 1.....	3.5	1.8	76.1	14.6
" " 2.....			39.6	7.6
" " 4.....	3.2	2.0	78.0	17.3
Brain, Rats 3 and 4.....	0.0	0.0	0.0	0.0

TABLE VI
Separation of Ethanolamine and Serine

Experiment No.	Serine fraction	Ethanolamine fraction
	c.p.m. per mg.	c.p.m. per mg.
1	1140	13.7
2	1137	25.0
3		3.7
4	1114	13.3
5	1218	16.8
6		42.6

Each sample contained 10 mg. of DL-serine with about 1100 c.p.m. per mg. and 4 mg. of inert ethanolamine.

The control experiments, by using inert ethanolamine with radioactive serine (Table VI), indicate that the procedure used does not separate the serine activity completely from the ethanolamine. However, on a molar basis a maximum of 1 per cent of the serine activity contaminated the ethanolamine. A comparison of Tables V and VI makes it apparent that the high activity of the ethanolamine from the liver lipides cannot be accounted for on the basis of contamination. Therefore, the β -carbon of serine must find its way to ethanolamine by a direct route. Stetten (3), using serine labeled with N¹⁵, found that the N¹⁵ appeared in the ethanola-

mine of the phospholipides in high concentration. He proposed a decarboxylation of serine to ethanolamine. Shemin (4) suggested a transformation to glycine by loss of the β -carbon, with a subsequent reduction of the carboxyl group as the pathway of serine to ethanolamine. The results obtained in these experiments are in harmony with the postulate of Stetten. The recent finding by Sakami (22) and also by Goldsworthy *et al.* (23) of the extremely rapid transformation of glycine to serine in conjunction with the present findings supports the assumption that the pathway of glycine to ethanolamine is via serine, according to the following scheme:



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SUMMARY

DL-Serine has been synthesized with C^{14} in the β -carbon. A method of assay of the specific activity of the β -carbon of serine based upon the titrimetric determination of methylenebismethone has been developed. Tracer experiments with serine- β - C^{14} have been carried out in intact rats. The results show a rapid uptake of the serine by the tissue proteins and the appearance of the β -carbon in ethanolamine. They, therefore, support the hypothesis that the pathway of glycine to ethanolamine is via serine.

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DESOXYRIBOSE-1-PHOSPHATE

I. THE PHOSPHOROLYSIS AND RESYNTHESIS OF PURINE DESOXYRIBOSE NUCLEOSIDE*

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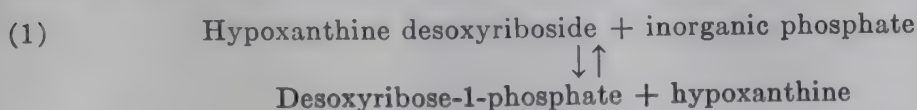
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This paper describes the isolation and properties of an extremely acid-labile phosphate ester which is considered to be the primary phosphorolysis product formed during the splitting of purine *desoxyribose* nucleosides. The experimental results presented in this paper together with those of Paper II (2) and Paper III (3) strongly indicate that this ester is *desoxyribose-1-phosphate*.

The phosphorolytic splitting of purine desoxyribose nucleosides appeared most likely in view of the finding that hypoxanthine and guanine ribosides could be split by enzymatic phosphorolysis with the formation of ribose-1-phosphate (4), a reaction analogous to the phosphorolysis of glycogen to glucose-1-phosphate (5). The phosphorolysis of hypoxanthine desoxyriboside, for example, can be considered to occur as follows:



In confirmation of Klein's early work on the splitting of desoxyribosides (6) it was noticed that crude nucleoside fractions obtained from hydrolysates of desoxyribose nucleic acid could be split by nucleoside phosphorylase from rat liver (7), with the formation of free hypoxanthine. The free hypoxanthine was determined spectrophotometrically by conversion to uric acid with milk xanthine oxidase (8). Manson and Lampen (9) have reported that they obtained the phosphorolysis and arsenolysis of hypoxanthine desoxyriboside by enzyme preparations from calf thymus and rat liver. Desoxyribose-5-phosphate was isolated as a product of phos-

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A preliminary report of this work has been published (1).

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phorolysis and evidence was obtained for its formation by a mutase type reaction from desoxyribose-1-phosphate. Manson and Lampen obtained further evidence for the formation of desoxyribose-1-phosphate during the phosphorolysis of thymidine (10). They found that the rate of formation of thymine from thymidine could be accelerated by the addition of hypoxanthine. This effect was in accord with the concept that desoxyribose-1-phosphate formed during the splitting of thymidine by pyrimidine nucleoside phosphorylase could react enzymatically with hypoxanthine (owing to the presence of purine nucleoside phosphorylase), and thus by removal of one of the end-products cause more thymidine to react. Wajzer in this laboratory (11) demonstrated the phosphorolysis of hypoxanthine desoxyriboside present in a hydrolysate of desoxyribose nucleic acid and showed that, upon the removal of inorganic phosphate in the incubation mixture, a resynthesis of the desoxyriboside occurred (as would be expected according to the reverse of Reaction 1).

The many observations which indicated that the desoxyribose nucleosides could be split by an enzymatic phosphorolysis analogous to that described for the ribose nucleosides made it quite probable that the primary phosphorolysis product could be obtained under proper conditions. This paper describes the isolation of an extremely acid-labile phosphate ester which is most specifically characterized by its ability to react enzymatically with hypoxanthine to form hypoxanthine desoxyriboside.

The isolation of the labile ester in pure and crystalline form (Paper II (2)), investigation of its properties, and microbiological data substantiating differential spectrophotometric evidence for the synthesis of desoxyribose nucleoside with the ester (Paper III (3)), all indicate that the phosphate ester can be best described as *desoxyribose-1-phosphate*.

The high instability of desoxyribose-1-phosphate is shown by the rapid dephosphorylation which occurs within a few minutes at pH 4 at room temperature. This is in marked contrast to the stability of ribose-1-phosphate at pH 4 (12). The dephosphorylation is accompanied by a loss in the ability of desoxyribose-1-phosphate to react enzymatically with hypoxanthine to form the desoxyriboside.

The possibility that only one enzyme is involved in the primary splitting of purine ribosides and desoxyribosides has been reinvestigated.

Preparation of Substrates and Enzymes

Guanine desoxyriboside was prepared according to a method described in Paper II (2). Hypoxanthine desoxyriboside was generously provided by Dr. Walter S. McNutt and Professor E. E. Snell of the University of Wisconsin. Xanthine oxidase was prepared according to the method of Ball (13). Inosine was prepared from adenosine by the action of adeno-

ne deaminase present in the alkaline phosphatase preparation from calf intestinal mucosa described by Schmidt and Thannhauser (14) (the enzyme preparation was carried through only one precipitation with ammonium sulfate).

Preparation of Calf Liver Nucleoside Phosphorylase—The enzyme was prepared according to the method described by Wajzer (15) with some modifications. Liver from a new-born calf¹ was perfused with cold tap water. The liver was then cut into small pieces and homogenized in a Waring blender with 2 volumes of cold distilled water. After centrifugation of the homogenate at 3000 r.p.m. in a refrigerated International centrifuge, the murky supernatant was poured off by decantation (volume = (A) ml.). To the supernatant fluid were added $0.1 \times (A)$ gm. of solid ammonium sulfate and also $0.43 \times (A)$ ml. of a saturated aqueous solution of ammonium sulfate (previously adjusted to pH 8.6 with 15 N NH_4OH). After standing overnight at 3° , the mixture was centrifuged at 10,000 r.p.m. at room temperature for 15 to 20 minutes. To the clear supernatant (volume = (B) ml.) was added $0.2 \times (B)$ gm. of solid ammonium sulfate. After standing overnight, the mixture was spun at 10,000 r.p.m. The protein precipitate was dissolved in a small volume of redistilled H_2O , and the solution transferred to a cellophane bag and dialyzed against several changes of redistilled H_2O (3°) for 2 days. After dialysis, a precipitate which had formed inside the bag was spun down and discarded. The clear supernatant enzyme solution was frozen and stored at -20° with very little loss of activity over a period of months.

The activity of the nucleoside phosphorylase from calf liver was expressed as micromoles of inosine split in 10 minutes upon addition of 25 $\mu\text{l.}$ of diluted enzyme to a standard assay solution containing 300 γ of inosine in 3.0 ml. of 0.1 M phosphate buffer, pH 8.0, and 5 $\mu\text{l.}$ of xanthine oxidase. In the presence of an excess of xanthine oxidase, the hypoxanthine released by the action of the nucleoside phosphorylase on inosine was immediately converted to uric acid, which in turn could be detected spectrophotometrically and quantitatively estimated by the increase of absorption at $\lambda = 293 \text{ m}\mu$ (8). The amount of protein was also estimated spectrophotometrically (16). According to the conditions of this assay, 1 mg. of protein in the calf liver preparation could catalyze the phosphorolysis of 1.8 μM of inosine within 10 minutes.

Preparation of Rat Liver Nucleoside Phosphorylase—The method of preparation of nucleoside phosphorylase from rat liver was essentially that described by Kalckar (7). 120 gm. of chilled liver from twenty adult rats were homogenized in a Waring blender with 2 volumes of cold water.

¹ We wish to thank Dr. J. Moustgaard of the Institute of Animal Physiology for his cooperation.

After centrifugation of the homogenate at 3000 r.p.m. (3°) the supernatant was poured off and adjusted to 0.4 S by the addition of 1.0 S. (1.0 S = saturated solution of ammonium sulfate at room temperature, 23° .) The precipitate was spun down and discarded. The supernatant was then adjusted to 0.6 S. The protein fraction, 0.4 S to 0.6 S, was centrifuged, washed with 0.6 S and dissolved in 30 ml. of redistilled H_2O . After dialysis in a cellophane bag against redistilled H_2O (3°) the enzyme solution was spun and the precipitate discarded. The clear supernatant was again adjusted to 0.4 S but this time with 1.0 S buffered at pH 7.0. (1.0 gm. of sodium phosphoglycerate, 0.11 gm. of cysteine hydrochloride, and 100 ml. of 1.0 S (5).) The protein fraction, 0.4 S to 0.6 S at pH 7.0, was collected, redissolved, dialyzed, and spun as described above. The nucleoside phosphorylase from rat liver prepared as above has approximately the same specific activity as the enzyme prepared from calf liver.

Preparation of Desoxyribose-1-phosphate in Solution—Guanine desoxyriboside was incubated with nucleoside phosphorylase in a Warburg vessel for 27 hours at 13° with components as follows: 8.0 mg. of guanine desoxyriboside, 50 mg. of Na_2HPO_4 , 2.0 ml. of 0.1 M alanylglycine buffer, pH 8.5, 40 μl . of catalase (0.25 mg. of protein), 100 μl . of xanthine oxidase (2.1 mg. of protein), 300 μl . of rat liver nucleoside phosphorylase (12 mg. of protein).

After completion of the incubation period, with a total oxygen uptake of $15.5 \mu\text{M}$, the vessel was chilled in an ice bath for 30 minutes. The mixture was then transferred to a centrifuge tube and the uric acid spun down and discarded. The supernatant was extracted three times with 2 ml. portions of cold *n*-butanol (saturated with water) and then extracted with ether to remove the butanol. 300 mg. of norit A were added and the mixture filtered through a pad of cotton and paper pulp. The filter pad was washed with 1.2 ml. of water. The clear filtrate was evaporated at room temperature with an air stream to approximately 0.8 ml. 3.0 ml. of magnesia mixture (5.0 ml. of 0.1 M MgSO_4 -1.0 M NH_4Cl and 0.1 ml. of 15 N NH_4OH) were added and the mixture then allowed to stand overnight in a refrigerator. The MgNH_4PO_4 was removed by filtering the mixture through a pad of cotton and paper pulp. The pad was washed with 0.5 ml. of magnesia mixture. The clear filtrate was evaporated at room temperature with an air stream to a volume of 2.7 ml. The solution contained $16 \mu\text{M}$ of labile P. The desoxyribose-1-phosphate solution was stored at -20° . Despite the denaturing effect of the butanol extractions, some preparations contained active xanthine oxidase. Xanthine oxidase could be completely denatured as follows: To 1.0 ml. of desoxyribose-1-phosphate solution were added 5.0 ml. of ethanol and the mixture allowed to stand overnight in a refrigerator. The alcohol was

then evaporated at room temperature with an air stream to a final aqueous volume of 1.0 ml. The insoluble precipitate which had formed was spun down and discarded. The desoxyribose-1-phosphate in solution thus isolated was used for the synthesis of desoxyriboside and for the demonstration of the presence of labile phosphorus.

Results

Accelerating Effect of Inorganic Phosphate on Rate of Splitting of Guanine Desoxyriboside by Nucleoside Phosphorylase—It has been demonstrated that nucleoside phosphorylase loses its enzymatic activity after prolonged dialysis and can be reactivated by the addition of inorganic phosphate (6, 4, 9, 11). This activation occurs when either ribosides or desoxyribosides are used as substrates. The effect of inorganic phosphate when guanine desoxyriboside serves as a substrate is described here in some detail as an introduction to the differential spectrophotometric methods which were used throughout these studies.

The action of nucleoside phosphorylase on guanine desoxyriboside can be followed spectrophotometrically owing to the following sequence of reactions. The nucleoside phosphorylase splits the desoxyriboside into free guanine and desoxyribose-1-phosphate. The free guanine is converted to xanthine owing to the presence of guanase in the nucleoside phosphorylase preparation. Xanthine oxidase, which is added in excess, converts xanthine to uric acid. Thus the splitting of guanine desoxyriboside can be immediately detected when the reaction is carried out in a Beckman quartz cuvette by following the increase of absorption at $\lambda = 293 \text{ m}\mu$, which is the characteristic maximum for uric acid in the ultra-violet band. This method of differential spectrophotometric analysis of purine compounds had been described in detail (8).

The increase of density at $\lambda = 293 \text{ m}\mu$ during the conversion of guanine desoxyriboside to uric acid was $\Delta \text{ density} = 9.01$ ($1 \text{ }\mu\text{M}$ of guanine desoxyriboside per ml. in 0.2 M phosphate buffer, pH 8.0, 1 cm. absorption cell). The value for guanine riboside was essentially the same, $\Delta \text{ density} = 9.06$.

As shown in Fig. 1, the splitting of guanine desoxyriboside occurred at a very slow rate when a dialyzed preparation of nucleoside phosphorylase from calf liver was used. However, upon the addition of $2 \text{ }\mu\text{M}$ of inorganic phosphate (which yielded a final concentration of 0.00067 M phosphate) a 40-fold increase in the velocity of the reaction resulted. The accelerating effect of inorganic phosphate could be demonstrated over a pH range of 6.9–3.5.

High Lability of Desoxyribose-1-phosphate at pH 4 at Room Temperature—Desoxyribose-1-phosphate is a phosphoric ester of extreme lability towards hydrogen ions. Thus, pH 4, which does not bring about signifi-

cant liberation of phosphate from a number of acid-labile esters (acetyl phosphate, phosphocreatine, and ribose-1-phosphate), causes rapid dephosphorylation of desoxyribose-1-phosphate even at room temperature. It was therefore impossible to show the formation of a phosphate ester by use of the Lowry-Lopez method (12) which had been applied to ribose-1-phosphate. The conditions of the Lowry-Lopez method are relatively

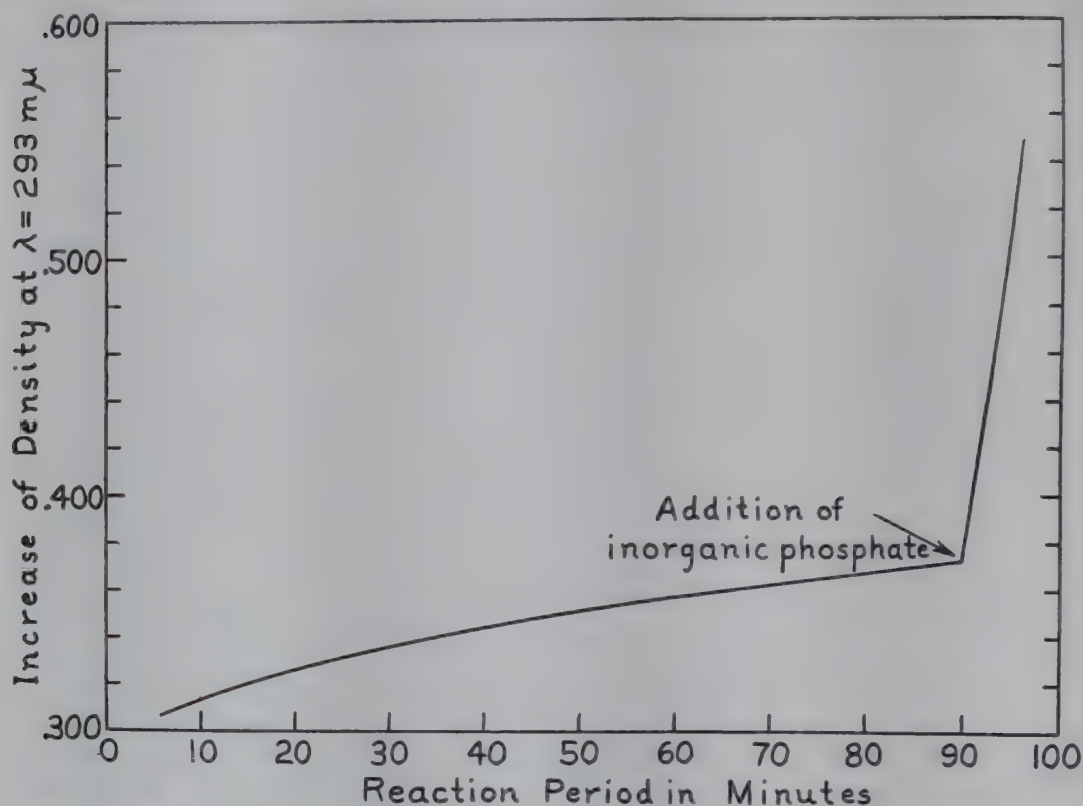


Fig. 1. Effect of inorganic phosphate on the rate of splitting of guanine desoxyriboside by nucleoside phosphorylase. The Beckman quartz cuvette contained 3.0 ml. of 0.05 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.75, 30 γ of guanine desoxyriboside, 20 $\mu\text{l.}$ of rat liver nucleoside phosphorylase, and 50 $\mu\text{l.}$ of xanthine oxidase. Inorganic phosphate was added as 30 $\mu\text{l.}$ of 0.067 M phosphate buffer, pH 7.0 (2 μM of P).

mild in that inorganic phosphate can be determined by color development at pH 4.

The presence of labile esterified phosphate in desoxyribose-1-phosphate was shown as follows: Desoxyribose-1-phosphate was added to acetate buffer at pH 4 at room temperature. After different time intervals the amount of inorganic phosphate present was determined by adjusting the pH of the buffer to 8.5 to 9.0 with 15 N NH_4OH and then precipitating the inorganic phosphate as MgNH_4PO_4 . As is indicated in Table I, a release of inorganic phosphate occurs when desoxyribose-1-phosphate stands in

acetate buffer. Approximately 50 per cent of the ester is destroyed by dephosphorylation within 10 to 15 minutes at room temperature. The high instability of desoxyribose-1-phosphate is in marked contrast to the stability of ribose-1-phosphate at pH 4.

Enzymatic Synthesis of Hypoxanthine Desoxyriboside—When desoxyribose-1-phosphate and hypoxanthine are incubated together with liver nucleoside phosphorylase in a phosphate-free medium, a rapid synthesis of hypoxanthine desoxyriboside occurs, according to the reverse of Reaction 1. This is shown by data from a typical experiment in Table II. The

TABLE I

Release of Inorganic Phosphate from Desoxyribose-1-phosphate at pH 4 (25°)

Aliquots of desoxyribose-1-phosphate in solution (0.1 ml.) were incubated at 25° with 0.2 ml. of 0.1 M acetate buffer, pH 4. Hydrolysis was stopped by the addition of 20 μ l. of 15 N NH_4OH . Because of the presence of excess Mg^{++} in the preparation, the inorganic phosphate released during hydrolysis of desoxyribose-1-phosphate was precipitated as MgNH_4PO_4 upon addition of NH_4OH . After standing overnight in a refrigerator, the mixture was centrifuged. P present in the precipitate was determined by the method of Gomori (17).

Time of hydrolysis	P pptd. as MgNH_4PO_4
<i>min.</i>	<i>γ of P</i>
0	0
5	6.1
10	10.8
15	13.5
20	15.6
30	17.6
40	19.3
60	19.4
120	17.5
180	20.1

method of spectrophotometric determination of free hypoxanthine and of desoxyribosidic bound hypoxanthine was the same as that used for resynthesis experiments with ribose-1-phosphate (4); *i.e.*, after heat inactivation of the incubation mixture and addition of phosphate buffer, first purified xanthine oxidase was added for the determination of free hypoxanthine and, second, nucleoside phosphorylase for the determination of hypoxanthine desoxyriboside.

As is shown in Table II, when all of the components were present, 47 per cent of the hypoxanthine was converted to the desoxyriboside within 30 minutes. In the absence of enzyme no reaction occurred between

desoxyribose-1-phosphate and hypoxanthine as indicated by complete recovery of hypoxanthine in the free form. When either desoxyribose-1-phosphate or hypoxanthine was omitted during the incubation period but added after heat inactivation of nucleoside phosphorylase, no formation of desoxyriboside could be detected.

These spectrophotometric data in evidence of synthesis of desoxyriboside were substantiated by microbiological assay. Hoff-Jørgensen has shown that *Thermobacterium acidophilus* R26 exhibits the same growth

TABLE II

Synthesis of Hypoxanthine Desoxyriboside with Desoxyribose-1-phosphate

The complete incubation mixture contained 0.2 ml. of desoxyribose-1-phosphate ($1.3 \mu\text{M}$ of labile P), 0.1 ml. of hypoxanthine ($0.15 \mu\text{M}$), 50 μl . of rat liver nucleoside phosphorylase, and 0.3 ml. of 0.1 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.55. Incubated at 30° for 30 minutes. The reaction was stopped by heating in a boiling water bath for 1 minute. Components omitted during incubation period were added after inactivation of the enzyme. In Experiment 4, enzyme was completely absent. Finally, 1.0 ml. of 0.2 M phosphate buffer, pH 8.0, was added. After cooling in a refrigerator for 2 hours, the mixtures from each experiment indicated in the table were centrifuged and the precipitates of coagulated protein and MgNH_4PO_4 (Mg^{++} and NH_4^+ present in the desoxyribose-1-phosphate preparation) discarded. The clear supernatants were subjected to differential spectrophotometric analysis for free hypoxanthine and desoxyribose nucleoside present.

Experiment No.	Description	Hypoxanthine (a)	Hypoxanthine desoxyriboside (b)	Total, (a) + (b)
		μM	μM	μM
1	Complete system	0.083	0.073	0.156
2	“ “ minus desoxyribose-1-phosphate	0.165	0	0.165
3	Complete system minus hypoxanthine	0.164	0	0.164
4	“ “ “ enzyme	0.155	0	0.155

response when equimolar amounts of the desoxyribosides of thymine, guanine, or hypoxanthine are added as specific growth factors (18, 19). The incubation mixtures from the four experiments in Table II were assayed microbiologically with *T. acidophilus* R26 (see Paper III (3)) for the presence of desoxyriboside. Only in the complete system (Experiment 1) could an appreciable amount of desoxyriboside be detected. A comparison of spectrophotometric and microbiological data is given in Paper III (3).

A dephosphorylation of desoxyribose-1-phosphate at pH 4 can be correlated with loss of its ability to react with hypoxanthine. As is shown by

the data in Table III, when desoxyribose-1-phosphate was allowed to stand in acetate buffer at pH 4 prior to its addition to hypoxanthine, no synthesis of desoxyriboside could be detected and all of the hypoxanthine originally added to the incubation mixture could be recovered as free hypoxanthine. In the control experiment with desoxyribose-1-phosphate not subjected to hydrolysis at pH 4, 47 per cent of the hypoxanthine was converted to the desoxyriboside.

Homogeneity of Purine Nucleoside Phosphorylase—It is of interest to ascertain whether purine nucleoside phosphorylase consists of a single enzyme or of two; that is, whether a single enzyme can catalyze the split-

TABLE III

Inactivation of Desoxyribose-1-phosphate at pH 4 (30°) As Indicated by Inability to React with Hypoxanthine

Experiment 1—0.2 ml. of desoxyribose-1-phosphate (1 μM of labile P) was added to 0.2 ml. of 0.1 M acetate buffer, pH 4. This mixture was allowed to stand at 30° for 20 minutes. The following components were then added: 20 $\mu\text{l.}$ of 1 N NaOH, 0.5 ml. of 0.1 M alanyl-glycine, pH 8.5, 0.2 ml. of hypoxanthine (0.14 μM), and 50 $\mu\text{l.}$ of rat liver nucleoside phosphorylase. The mixture was incubated at 30° for 30 minutes, then placed in a boiling water bath for 1 minute. After inactivation of the enzyme at 100°, 0.6 ml. of 0.2 M phosphate buffer, pH 8.0, was added. Further treatment was the same as that described in Table II.

Experiment 2—Same as Experiment 1 except for order of addition of the components. NaOH was added prior to addition of acetate buffer and hence desoxyribose-1-phosphate was at no time subjected to hydrolysis at pH 4.0.

Experi- ment No.	Description	Hypoxan- thine (a)	Hypoxan- thine des- oxyriboside (b)	Total, (a) + (b)
		μM	μM	μM
1	Desoxyribose-1-phosphate, pH 4	0.139	0	0.139
2	“ not hydrolyzed	0.076	0.066	0.142

ting of purine riboside as well as purine desoxyriboside. Klein (6) has concluded that only one enzyme is involved, mainly because he found that nucleoside phosphorylase preparations with varying degrees of purity always split the riboside and desoxyriboside with approximately equal velocity. With the development of differential spectrophotometry it is now possible to examine this problem more closely; however, absolute proof that a single enzyme is involved must await the isolation of nucleoside phosphorylase in pure form.

If a single enzyme is involved, then with two substrates which are as similar in structure as hypoxanthine riboside and hypoxanthine desoxyriboside one would expect the Michaelis-Menten constants (20) to be es-

essentially equal. Kinetic studies were therefore carried out with nucleoside phosphorylase from calf liver and the two substrates and in each case the effect of the concentration of the substrate on the velocity of the enzyme-catalyzed reaction was determined. In view of the fact that the rate of splitting was determined by conversion of hypoxanthine to uric acid in the presence of xanthine oxidase, care was taken to add xanthine oxidase in excess so as not to make the oxidation of free hypoxanthine the limiting factor.

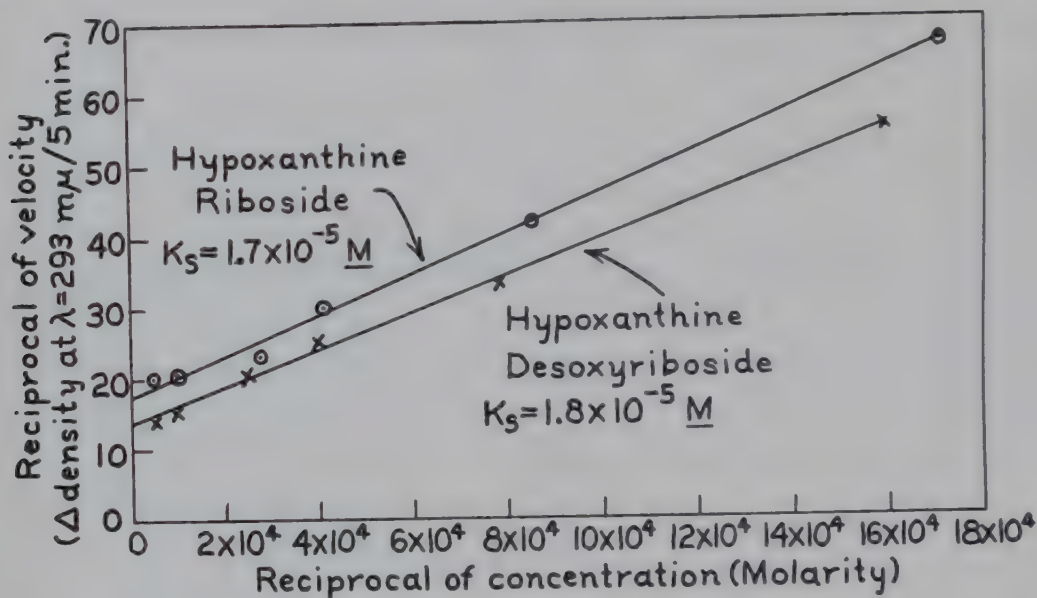


FIG. 2. Michaelis-Menten constants of calf liver nucleoside phosphorylase with two substrates, hypoxanthine riboside and hypoxanthine desoxyriboside. The Beckman cuvette contained 3.0 ml. of 0.1 M phosphate buffer, pH 7.4, 20 μ l. of xanthine oxidase, 0.1 ml. of substrate (160, 80, 40, 20, 10, and 5 γ of hypoxanthine riboside or hypoxanthine desoxyriboside), and 25 μ l. of calf liver nucleoside phosphorylase. The increase of density at $\lambda = 293 \text{ m}\mu$ was followed during a 5 minute interval (2 to 7 minutes) after addition of the nucleoside phosphorylase. Temperature, 24°.

As is shown in Fig. 2, when the reciprocal of the velocity was plotted *versus* the reciprocal of the substrate concentration according to the method of Lineweaver and Burk (21), a straight line was obtained for each substrate. The Michaelis-Menten constants were found to be almost identical: hypoxanthine riboside, $1.7 \times 10^{-5} \text{ M}$; hypoxanthine desoxyriboside, $1.8 \times 10^{-5} \text{ M}$.

It is conceivable that, although the constants are the same within experimental error, separate enzymes are operating. In this case if hypoxanthine riboside were present at a concentration which yields maximum velocity, then the addition of hypoxanthine desoxyriboside (in the presence

of a separate enzyme) should still further increase the velocity as measured by the rate of formation of uric acid. Actually, when this experiment was carried out, essentially the same velocity was observed when the riboside and desoxyriboside were present separately or together in the incubation mixture at concentrations which were equivalent to maximum velocity for each substrate. These results are in accord with the hypothesis that only one enzyme is present, that it is completely saturated with respect to one substrate, and is therefore operating at maximum velocity, and that upon the addition of the second substrate which has the same affinity for the enzyme as the first substrate (same Michaelis-Menten constant), no significant increase of velocity can occur. Considerations of the kinetics of a system (one enzyme with two different substrates) have recently been published (22, 23).

Although these kinetic experiments are by no means definitive, they strongly hint that the same enzyme can catalyze the phosphorolysis of hypoxanthine riboside and of hypoxanthine desoxyriboside, thus substantiating Klein's earlier work.

SUMMARY

The enzymatic phosphorolysis of guanine desoxyriboside yields a labile phosphate ester which is characterized by its ability to react with hypoxanthine in the presence of nucleoside phosphorylase to form hypoxanthine desoxyriboside. The ester is rapidly dephosphorylated at pH 4 at room temperature and at the same time loses its property of forming desoxyriboside. Kinetic studies yield additional evidence for Klein's belief that nucleoside phosphorylase, as a single entity, can split both inosine and hypoxanthine desoxyriboside.

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DESOXYRIBOSE-1-PHOSPHATE

II. THE ISOLATION OF CRYSTALLINE DESOXYRIBOSE-1-PHOSPHATE

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The formation of an extremely acid-labile phosphate ester during the enzymatic phosphorolysis of guanine desoxyriboside has been demonstrated in Paper I (1). The ester was most specifically characterized by its ability, in the presence of nucleoside phosphorylase, to react with hypoxanthine and form hypoxanthine desoxyriboside. The formation of hypoxanthine desoxyriboside was substantiated by two separate approaches: by differential spectrophotometry and by microbiological assay which indicated that the enzymatically produced desoxyriboside could act as a growth factor for *Thermobacterium acidophilus* R26 (see Paper III (2)). The phosphate ester, when subjected to very mild hydrolysis at pH 4, released inorganic phosphate and at the same time lost its ability to react with hypoxanthine. On the basis of these experiments it appeared very probable that the ester formed during the phosphorolysis of guanine desoxyriboside was *desoxyribose-1-phosphate*.

This paper deals with the isolation and properties of desoxyribose-1-phosphate obtained in pure form as a crystalline salt of the organic base, cyclohexylamine.

Crystalline guanine desoxyriboside prepared from thymus nucleic acid was incubated with nucleoside phosphorylase from calf liver. The free guanine was converted to insoluble xanthine owing to the presence of guanase in the enzyme preparation. After extraction of the incubation mixture with *n*-butanol, inorganic phosphate was removed with magnesia mixture and the barium salt of desoxyribose-1-phosphate precipitated by the addition of ethanol. The salt was further purified by reprecipitation from wet butanol. The cyclohexylamine salt was obtained from the barium salt by crystallization from a butanol-ether-water mixture after removal of barium as BaSO₄. The use of cyclohexylamine was suggested by Dr. Malcolm Clark of the University of Cambridge.

The phosphate ester isolated as the barium salt contains 1 mole of

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desoxyribose per mole of phosphorus. Of the total phosphorus present in the barium salt, 90 per cent appears as inorganic phosphate by the method of Gomori (3); yet the salt contains no phosphate precipitable as MgNH_4PO_4 unless first subjected to hydrolysis at pH 4. The lability of the phosphate linkage points to the aldehyde carbon of desoxyribose as the site of esterification. This is further substantiated by the release of free aldehyde at pH 4 as determined by iodometric titration.

The cyclohexylamine and barium salts of desoxyribose-1-phosphate can react directly in the presence of nucleoside phosphorylase with hypoxanthine to form hypoxanthine desoxyriboside as evidenced by spectrophotometric and microbiological data.

The equilibrium constant for the phosphorolysis of hypoxanthine desoxyriboside has been determined by approaching the equilibrium point from both sides and found to be equal to the constant for the phosphorolysis of inosine (4).

EXPERIMENTAL

Preparation of Guanine Desoxyriboside—19.6 gm. of nucleic acid (sodium salt, from thymus gland; British Drug Houses, Ltd., London) were dissolved in 200 ml. of 1 N NaOH and incubated at 33° for 20 hours in order to free the preparation of pentose nucleic acid (5). 400 ml. of water were then added and the pH adjusted to 6.8 by the addition of 12 N HCl (chilled solutions). The mixture was filtered and the nucleic acid precipitated according to the method of Hammarsten (6).

The nucleic acid was enzymatically degraded to nucleosides as follows: 11.5 gm. of the reprecipitated nucleic acid (free acid) were slowly added to a mixture of 400 ml. of water and 7.0 ml. of 15 N NH_4OH in a Waring blender; 50 ml. of a saturated solution of ammonium sulfate and 24.6 gm. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were also added. The homogeneous mixture was washed into a beaker with 100 ml. of water and the pH adjusted to 8.7 by the addition of 3.0 ml. of 15 N NH_4OH . 150 ml. of intestinal mucosal phosphatase¹ were added. The mixture was incubated for 3 days at 33° with toluene added as preservative. The enzymatic dephosphorylation of the nucleic acid was followed during this period by deproteinizing an aliquot of the incubation mixture with cold perchloric acid and then determining the amount of purine desoxyribosides present by the spectrophotometric methods of Kalckar (9).

After termination of the incubation period, the mixture was filtered and extracted three times with a total of 10 volumes of *n*-butanol saturated

¹ The enzyme was prepared according to the procedure of Schmidt and Thannhauser (7) and consisted of the protein precipitated by the first addition of ammonium sulfate. The combined phosphatase and adenosinedeaminase activity of the preparation was determined by following spectrophotometrically the rate of formation of inosine from adenosine-5-phosphate in the presence of the enzyme (8).

with water. The butanol extract was filtered and then distilled *in vacuo* (temperature of water bath, 40–45°). Water was added at intervals during the distillation to maintain the azeotrope (60 per cent butanol-40 per cent water). When all of the butanol had been displaced by several additions of water, the final aqueous residue, 70 ml., was filtered and placed in a refrigerator. A heavy gel formed overnight. The gel was spun down in a refrigerated centrifuge at 10,000 r.p.m., washed with two small portions of cold water, and then redissolved in water at 50°. Upon cooling, the solution again yielded a gel which was spun down in the cold. After two such precipitations as described above, guanine desoxyriboside appeared in crystalline form. After two more recrystallizations, the desoxyriboside was collected and dried *in vacuo* at room temperature. The crystallinity was corroborated by x-ray diffraction.² Ultraviolet spectral changes with change in pH were found to be the same as those reported by Hotchkiss (10). The preparation was shown to be free of hypoxanthine desoxyriboside by hydrolyzing a small portion in dilute acid and then incubating with xanthine oxidase. No formation of uric acid could be detected, as would be the case if hypoxanthine were present.

Preparation of Barium Salt of Desoxyribose-1-phosphate—The phosphorolysis of guanine desoxyribose was carried out by incubating the following mixture at 24° for 24 hours: 100 mg. of guanine desoxyriboside, 600 mg. of Na_2HPO_4 , 24 ml. of 0.1 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.5, 3.6 ml. of calf liver nucleoside phosphorylase (see Paper I (1)).

After incubation, the mixture was cooled in an ice water bath and then centrifuged (the guanase present in the enzyme preparation converts guanine to xanthine, which precipitates during the incubation period). The supernatant was extracted three times with 85 ml. portions of *n*-butanol saturated with water. The layers were separated by centrifugation. Denatured protein appeared at the interface. To the aqueous layer were added 14.4 ml. of 0.5 M MgCl_2 -5.0 M NH_4Cl and 1.2 ml. of 15 N NH_4OH . After 3 hours in a refrigerator, the MgNH_4PO_4 precipitate was removed by filtration through a pad of cotton and paper pulp. To the clear filtrate were added 3.6 ml. of ammoniacal barium acetate (2.0 gm. of $\text{Ba}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$, 10 ml. of H_2O , and 0.6 ml. of 15 N NH_4OH). The mixture was placed in a refrigerator. The precipitate which formed overnight was spun down and discarded. To the clear supernatant were added 4 volumes of ethanol and 0.4 ml. of 15 N NH_4OH . After 10 hours in the refrigerator, the barium salt which had formed was centrifuged, washed two times by suspension in ethanol, and finally freed of alcohol by suspension in diethyl ether. The salt was dried *in vacuo* at room temperature.

² X-ray diffraction analyses were kindly provided by Dr. Tovborg-Jensen and Dr. Knækgergaard of the Royal Veterinary School.

The crude barium salt of desoxyribose-1-phosphate was reprecipitated as follows: 95 mg. of the crude salt were extracted three times with 2.0 ml. portions of cold water. After three such extractions an insoluble residue was left after centrifugation and was discarded. To the water extracts were added 15 volumes of dry butanol which, upon stirring, yielded a one-phase system owing to the solubility of water in butanol. The barium salt which precipitated upon the addition of butanol was spun down, washed three times with diethyl ether, and dried *in vacuo* at room temperature. The final yield of purified barium salt, starting with 100 mg. of guanine desoxyriboside, was 15 mg.

Preparation of Cyclohexylamine Salt of Desoxyribose-1-phosphate—35.7 mg. of the purified barium salt of desoxyribose-1-phosphate were dissolved in 400 μ l. of H_2O . 23.5 mg. of cyclohexylamine sulfate in 100 μ l. of H_2O were added. (Cyclohexylamine sulfate, $(C_6H_{11}NH_2)_2 \cdot H_2SO_4$, was prepared as follows: To 1.0 ml. of cyclohexylamine dissolved in 5 ml. of ethanol were added 8.7 ml. of alcoholic H_2SO_4 (1.0 ml. of 10 $\times$ H_2SO_4 + 9.0 ml. of absolute ethanol). A crystalline precipitate of cyclohexylamine sulfate formed immediately. The salt was washed with ethanol and dried *in vacuo* at room temperature.) The precipitate of $BaSO_4$ was spun down. The clear supernatant was pipetted into 7.5 ml. of absolute *n*-butanol. This mixture was vigorously stirred until all the water had dissolved in the organic solvent, and then placed in a refrigerator overnight. A small amount of precipitate was spun down and discarded. To the clear supernatant was added approximately an equal volume of diethyl ether. After standing at room temperature for 15 to 20 minutes, the butanol-ether-water mixture yielded a mass of fine long needles. The crystals were collected, washed with ether, and then redissolved in 0.2 ml. of water. 3.0 ml. of dry *n*-butanol were added. The butanol-water mixture was filtered through a sintered glass disk. 1.0 ml. of ether was then added gradually until the solution was faintly cloudy. After standing at room temperature for 1 hour the solution was filled with a mass of fine long needles. The cyclohexylamine salt was collected, washed with ether, and dried *in vacuo* at room temperature. 7.0 mg. of recrystallized salt were obtained. Qualitative tests indicated the presence of desoxyribose (Dische cysteine- H_2SO_4 color test modified by Stumpf (11)) and inorganic P and the absence of sulfate. A photomicrograph of the crystalline salt is shown in Fig. 1. The salt sinters and starts to decompose at 152°.

$C_{17}H_{37}O_7N_2P$	Found.	P 7.34, N 6.75 ³
412.26	Calculated.	" 7.52, " 6.79

P was determined as inorganic P at pH 1 by the method of Gomori (3)

³ The micro-Kjeldahl analysis was kindly provided by Søren Løvtrup, Cytochemical Department of the Carlsberg Laboratories.

Analysis of Barium Salt of Desoxyribose-1-phosphate—When analyzed for desoxyribose and phosphorus, the barium salt was found to contain these two constituents in equimolar amounts. 1.00 mg. of the barium salt was found to be equivalent to des-oxyribose,⁴ 2.0 μM (hypoxanthine des-oxyriboside as standard), 2.2 μM (guanine desoxyriboside as standard),



FIG. 1. Cyclohexylamine salt of des-oxyribose-1-phosphate. $\times 92$. Kindly photographed by G. G. Knappeis.

and phosphorus, 2.0 μM as total phosphorus, 1.8 μM as inorganic phosphorus.⁵

According to the analytical data 90 per cent of the total phosphorus in the barium salt appears as inorganic phosphate (determined at pH 1). This indicates that only a small portion of the phosphate present can be

⁴ Desoxyribose was determined according to the method of Stumpf (11).

⁵ Determined according to the method of Gomori (3).

in the form of a stable alcoholic phosphate ester. On the other hand the fact that most of the phosphate is actually bound in the form of a highly labile ester is illustrated by the data in Table I. The barium salt when dissolved in acetate buffer at pH 4 contains no inorganic phosphate pre-

TABLE I

Release of Inorganic Phosphate from Barium and Cyclohexylamine Salts of Desoxyribose-1-phosphate at pH 4

3.066 mg. of barium desoxyribose-1-phosphate were dissolved in 250 μ l. of H_2O . To 25 μ l. aliquots of this solution in a series of separate centrifuge tubes were added 200 μ l. of 0.1 M acetate buffer, pH 4. The period of hydrolysis at 21° (different interval of time for each tube as indicated in the table) was terminated by the addition of 20 μ l. of 15 N NH_4OH . In the zero time tube, NH_4OH was added prior to the acetate buffer. Each mixture was chilled in an ice bath and then 30 μ l. of magnesia mixture (0.5 M $MgCl_2$ -5.0 M NH_4Cl) were added. After 8 hours at 2°, the tubes were centrifuged and the supernatants carefully withdrawn. The P present in the precipitate was determined directly according to Gomori (3). The same procedure was followed with the cyclohexylamine salt, starting with 2.50 mg. of the salt in 200 μ l. of H_2O (hydrolysis at 23°).

Time	Inorganic P pptd. as $MgNH_4PO_4$	Hydrolysis
Ba salt; each aliquot contained total P 19 γ , inorganic P (determined at pH 1) 17 γ		
min.	γ	per cent
0	0	0
5	2.7	16
10	6.6	39
15	8.3	49
22	9.9	58
30	12.2	72
40	11.4*	67*
60	15.3	90

Cyclohexylamine salt; each aliquot contained 21.8 γ of esterified P

0	0	0
5	4.4	20
15	11.1	51
27	15.9	73
52	17.9	82
81	21.3	98

cipitable as $MgNH_4PO_4$ at zero time, but as the hydrolysis proceeds at room temperature, an increasing amount of inorganic phosphate precipitable with magnesia mixture is released, approaching the value for total inorganic phosphate as determined by the Gomori method (3).

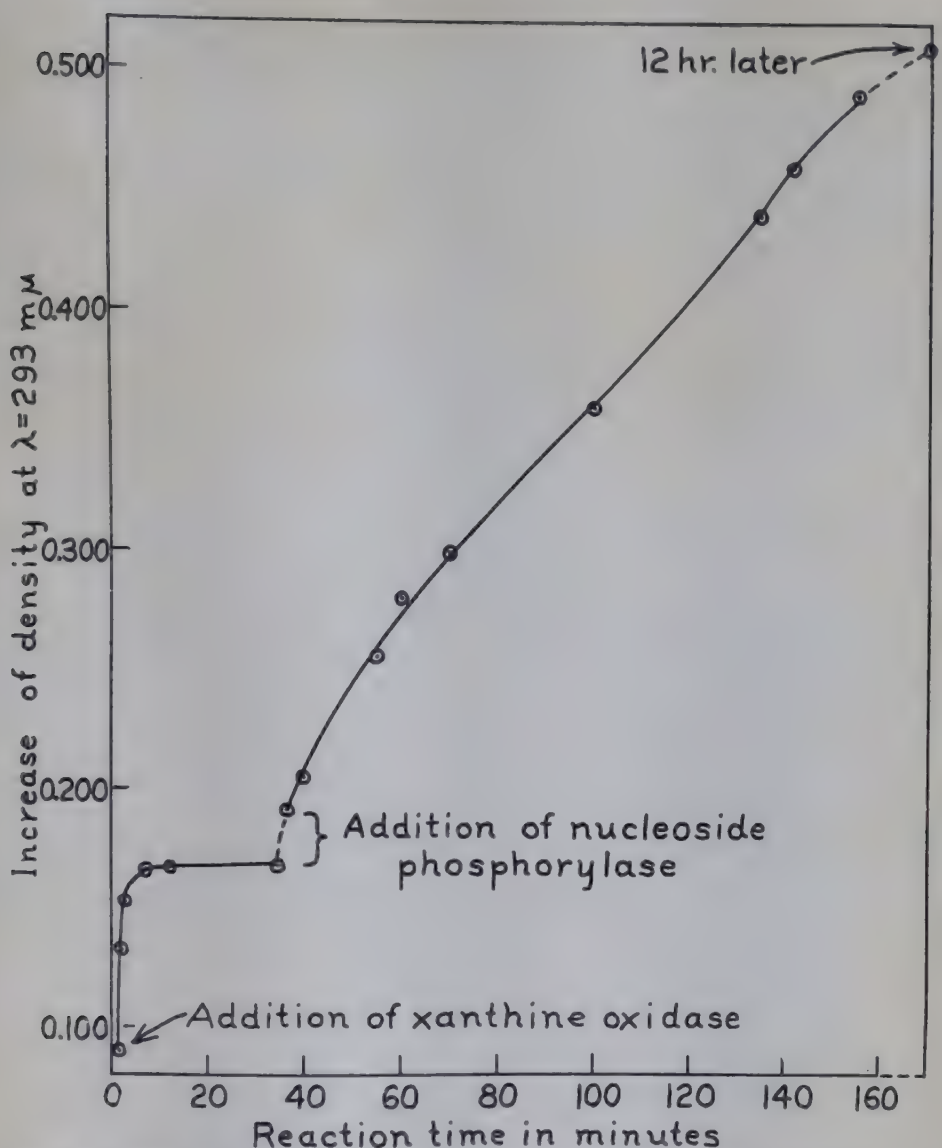


FIG. 2. Synthesis of desoxyriboside; differential spectrophotometric analysis of a mixture containing hypoxanthine and hypoxanthine desoxyriboside. 25 μl . of calf liver nucleoside phosphorylase (0.4 mg. of protein) were added to a mixture consisting of 1.23 mg. of the barium salt of desoxyribose-1-phosphate and 272 γ of hypoxanthine in 500 μl . of 0.08 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.4. After 30 minutes incubation at 24°, a 50 μl . aliquot was taken and the enzyme therein inactivated by heating for 2 minutes at 100°. 5.0 ml. of 0.1 M phosphate buffer, pH 7.4, were added and the coagulated protein removed by filtration. To 3.0 ml. of the clear filtrate in a Beckman quartz cuvette were added, first, 15 μl . of xanthine oxidase and, second, 15 μl . of calf liver nucleoside phosphorylase (50 γ of protein).

A similar release of inorganic phosphate could be demonstrated with the crystalline cyclohexylamine salt, as shown in Table I.

Release of Free Aldehyde at pH 4—The evidence for the presence of a highly labile phosphate linkage points to the aldehyde carbon of desoxy-

ribose as the site of phosphorylation. This can be tested by showing that free aldehyde is released as well as inorganic phosphate when desoxyribose-1-phosphate is hydrolyzed.

After hydrolysis of the barium salt in 0.1 M acetate buffer, pH 4 (25°), the pH was adjusted to 9.9 by the addition of an equal volume of 5 per cent Na_2CO_3 . The iodometric method of MacLeod and Robison (12), adapted for microtitration, was then applied for the estimation of free aldehyde. Although a significant increase in the consumption of iodine correlated with the time of hydrolysis could be demonstrated, a mole per mole relationship between aldehyde and phosphate liberated could not be definitely

TABLE II

Synthesis of Hypoxanthine Desoxyriboside with Crystalline Cyclohexylamine Desoxyribose-1-phosphate

25 μl . of calf liver nucleoside phosphorylase (0.4 mg. of protein) were added to a mixture consisting of 2.5 μM of cyclohexylamine desoxyribose-1-phosphate (1.03 mg.) and 2.5 μM of hypoxanthine in 400 μl . of 0.1 M tris(hydroxymethyl)amino-methane-HCl buffer, pH 7.4; temperature, 23.5°. Aliquots of 50 μl . each were taken at intervals as indicated and the enzymatic reaction stopped by heating for 2 minutes at 100°. 5 ml. of 0.1 M phosphate buffer, pH 7.7, were added to the heat-inactivated aliquot and, after filtration, the clear supernatant was subjected to spectrophotometric analysis for hypoxanthine desoxyriboside formed.

Time	Formation of hypoxanthine desoxyriboside	Conversion of hypoxanthine to desoxyriboside
<i>min.</i>	μM	<i>per cent</i>
2	0.2	8
10	0.7	28
23	1.4	56
45	1.9	76
61	2.1	84

established. The iodometric method could not be used directly with the cyclohexylamine salt of desoxyribose-1-phosphate owing to a reaction between the organic base and alkaline iodine.

Enzymatic Synthesis of Hypoxanthine Desoxyriboside

The synthesis of hypoxanthine desoxyriboside by the enzymatic reaction between hypoxanthine and desoxyribose-1-phosphate in solution has been demonstrated and discussed in Paper I (1). This work has been more fully extended by the isolation of desoxyribose-1-phosphate in the form of barium and cyclohexylamine salts. Both salts can be used directly in resynthesis experiments because barium and cyclohexylamine in small amounts do not inhibit nucleoside phosphorylase.

Fig. 2 illustrates differential spectrophotometric data obtained in a typical synthesis with the barium salt of desoxyribose-1-phosphate and hypoxanthine. $2.3 \mu\text{M}$ of the barium salt and $2.0 \mu\text{M}$ of hypoxanthine were incubated in the presence of nucleoside phosphorylase. The enzyme was

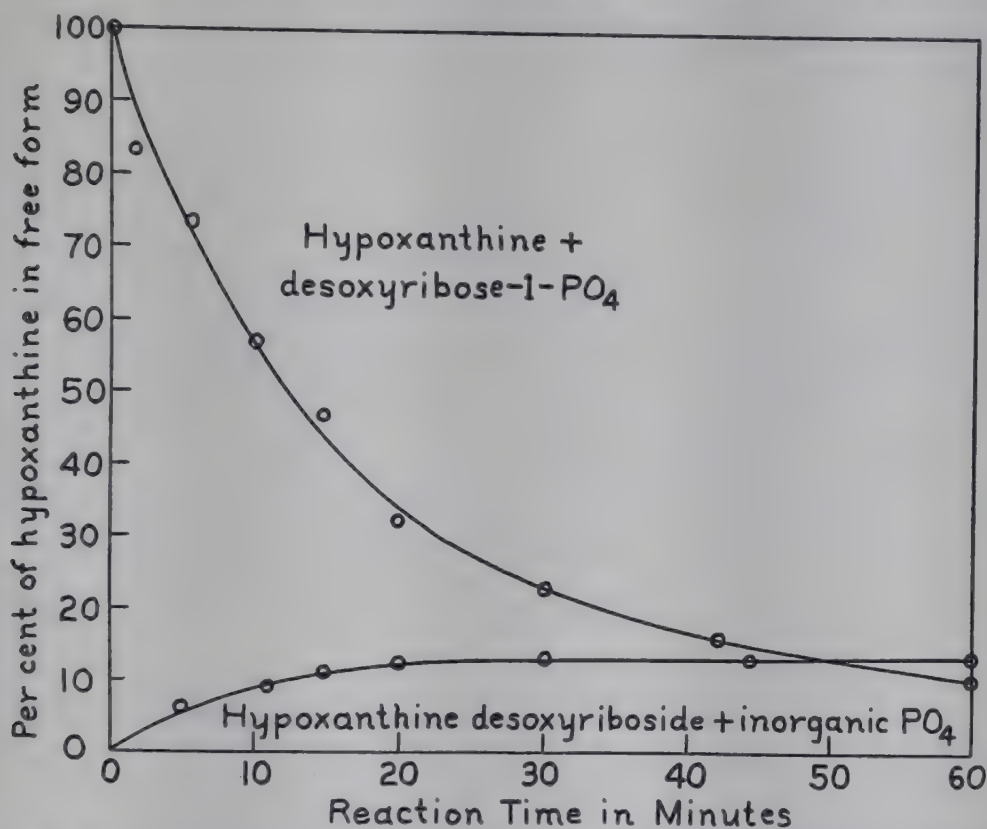


FIG. 3. Equilibrium constant for phosphorolysis of hypoxanthine desoxyriboside. *Phosphorolysis*, $2.2 \mu\text{M}$ of inorganic P and $2.2 \mu\text{M}$ of hypoxanthine desoxyriboside in $500 \mu\text{l.}$ of 0.03 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.4. $25 \mu\text{l.}$ of calf liver nucleoside phosphorylase (0.4 mg. of protein) were added at zero time; temperature, 24° . Aliquots of $50 \mu\text{l.}$ each were taken at intervals as indicated and the enzymatic reaction stopped by heating for 2 minutes at 100° . 5.0 ml. of 0.1 M phosphate buffer, pH 7.4, were added to the heat-inactivated aliquot and after filtration the sample was subjected to spectrophotometric analysis for the amount of free hypoxanthine present. *Synthesis*, $2.2 \mu\text{M}$ of desoxyribose-1-phosphate and $2.2 \mu\text{M}$ of hypoxanthine in $500 \mu\text{l.}$ of buffer as above. Further treatment was the same as described above. The desoxyribose-1-phosphate used for this experiment was prepared as follows: 2.25 mg. of the barium salt were dissolved in $300 \mu\text{l.}$ of H_2O . Barium was removed by the addition of 5 mg. of Na_2SO_4 in $50 \mu\text{l.}$ of H_2O . $200 \mu\text{l.}$ of the clear supernatant contained $2.2 \mu\text{M}$ of phosphate, determined by the method of Gomori (3).

then heat-inactivated, phosphate buffer added, and the following additions carried out in a Beckman quartz cuvette. First, xanthine oxidase was added. The increase of density at $\lambda = 293 \text{ m}\mu$ was then followed. A plateau was very quickly reached, as indicated in Fig. 2. This first

increase was due to the presence of free hypoxanthine. Upon the second addition of enzyme, in this case, nucleoside phosphorylase from calf liver, the density of the solution again increased and eventually attained a new and higher plateau. This increase was due to the release of hypoxanthine from hypoxanthine desoxyriboside formed during the primary enzymatic incubation of hypoxanthine and the barium salt. In this experiment 76 per cent of the hypoxanthine initially present was converted to the desoxyriboside in 30 minutes. Microbiological assay with *T. acidophilus* R26 (see Paper III (2)) also indicated the formation of desoxyriboside in other experiments with the barium salt of desoxyribose-1-phosphate.

Similar syntheses were carried out with cyclohexylamine desoxyribose-1-phosphate. In Table II are data for the formation of desoxyriboside as a function of the time of incubation when equimolar amounts of the crystalline cyclohexylamine salt and hypoxanthine were used. After 1 hour 84 per cent of the hypoxanthine initially present was converted to the desoxyriboside. Microbiological assay again corroborated these results. These experiments with desoxyribose-1-phosphate are completely analogous to those described by Kalckar with ribose-1-phosphate (4).

Equilibrium Constant for Phosphorolysis of Hypoxanthine Desoxyriboside

Essentially the same equilibrium constant ($K_{\text{phosphorolysis}} = 1/54$) was obtained for the phosphorolysis of hypoxanthine desoxyriboside when the equilibrium was approached from both sides, as shown in Fig. 3. It is the synthesis rather than the splitting of the desoxyriboside which is favored, for at equilibrium 88 per cent of the hypoxanthine is present as nucleoside, starting with equimolar quantities of hypoxanthine and desoxyribose-1-phosphate. The same value was obtained by Kalckar (4) for the phosphorolysis of inosine.

SUMMARY

Desoxyribose-1-phosphate, an extremely acid-labile ester formed by the enzymatic phosphorolysis of guanine desoxyriboside, has been isolated as a crystalline salt of the organic base, cyclohexylamine.

Analytical data indicate the presence of 1 mole of desoxyribose per mole of phosphorus. The salt contains no phosphate precipitable as MgNH_4PO_4 unless first subjected to hydrolysis at pH 4. 50 per cent of the phosphorus is released as inorganic phosphate within 10 to 15 minutes upon hydrolysis at pH 4 at 23°. Free aldehyde, determined by iodometric titration, is also released upon hydrolysis at pH 4. On the basis of origin analyses, and properties the ester is designated as desoxyribose-1-phosphate.

Hypoxanthine desoxyriboside is synthesized when cyclohexylamine o

barium salts of desoxyribose-1-phosphate are incubated with hypoxanthine in the presence of nucleoside phosphorylase. The formation of desoxyriboside is shown by spectrophotometric and microbiological data.

Determination of the equilibrium constant, $K_{\text{phosphorolysis}} = 1/54$, indicates that the synthesis rather than the splitting of hypoxanthine desoxyriboside is favored.

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DESOXYRIBOSE-1-PHOSPHATE

III. COMPARISON OF MICROBIOLOGICAL AND SPECTROPHOTOMETRIC ESTIMATIONS OF ENZYMATICALLY PRODUCED PURINE DESOXYRIBOSE NUCLEOSIDE

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A newly developed method for the microbiological assay of desoxyribose nucleosides (1, 2) has been used to substantiate differential spectrophotometric evidence (3-5) for the formation of desoxyriboside when desoxyribose-1-phosphate and hypoxanthine are incubated in the presence of nucleoside phosphorylase.

The bacterium used for the microbiological assay was *Thermobacterium acidophilus* R26 (Orla-Jensen collection) which specifically requires desoxyribosides for growth. Pure hypoxanthine desoxyriboside, prepared by Dr. W. S. McNutt and two thymidine preparations¹ were used as standards. A description of the assay medium has been published elsewhere (2).

Results

As is shown in Table I (Experiment 1), both the microbiological and spectrophotometric data demonstrate that a significant synthesis of desoxyriboside occurs when hypoxanthine and desoxyribose-1-phosphate are incubated with nucleoside phosphorylase. When any of the components of the complete system were omitted during the incubation and added after heat inactivation of the enzyme, no formation of desoxyriboside could be detected. The impure desoxyribose-1-phosphate used in this experiment (Table I) appeared to contain a small amount of desoxyriboside.

The synthesis of desoxyriboside with the isolated barium salt of desoxyribose-1-phosphate was detected by microbiological assay as follows:

Experiment 1—The incubation mixture consisted of 50 μ l. of hypoxan-

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¹ Kindly supplied by Dr. W. Shive, University of Texas, and Dr. R. Hotchkiss, The Rockefeller Institute for Medical Research (Levene collection).

thine ($0.15 \mu\text{M}$), $50 \mu\text{l.}$ of the barium salt of desoxyribose-1-phosphate (380γ), $50 \mu\text{l.}$ of rat liver nucleoside phosphorylase, and $50 \mu\text{l.}$ of 0.1 M alanyl-glycine buffer, pH 8.5. The mixture was incubated for 30 minutes at 30° , and then placed in a boiling water bath for 1 minute.

Microbiological assay indicated the formation of $0.13 \mu\text{M}$ of desoxyriboside, a conversion of 87 per cent of the hypoxanthine initially present to the nucleoside.

TABLE I

*Comparison of Microbiological and Spectrophotometric Determinations of Enzymatically Produced Desoxyriboside**

The incubation mixture consisted of $100 \mu\text{l.}$ of hypoxanthine ($0.15 \mu\text{M}$), $200 \mu\text{l.}$ of desoxyribose-1-phosphate in solution ($1.3 \mu\text{M}$ of labile P), $50 \mu\text{l.}$ of rat liver nucleoside phosphorylase, and $300 \mu\text{l.}$ of 0.1 M tris(hydroxymethyl)aminomethane-HCl, buffer pH 8.55. The mixture was incubated for 30 minutes at 30° , and then placed in a boiling water bath for 1 minute.

Experiment No.	Description	Microbiological assay of desoxyriboside formed	Spectrophotometric data		
			Free hypoxanthine (a)	Desoxyriboside (b)	Total, (a) + (b)
		μM	μM	μM	μM
1	Complete system	0.083	0.083	0.073	0.156
2	“ “ minus desoxyribose-1-phosphate	0.012	0.165	0	0.165
3	Complete system minus hypoxanthine	0.012	0.164	0	0.164
4	Complete system minus enzyme	0.013	0.155	0	0.155
5	Desoxyribose-1-phosphate alone	0.012			

* See Table II, Paper I (4).

Experiment 2—This is the same as Experiment 1 except that the barium salt of desoxyribose-1-phosphate was not added until after heat inactivation of the enzyme.

In this case microbiological assay showed the formation of less than $0.003 \mu\text{M}$ of desoxyriboside compared to $0.13 \mu\text{M}$ for the complete active system.

When equimolar amounts of the crystalline cyclohexylamine salt of desoxyribose-1-phosphate and hypoxanthine were incubated with the enzyme (Table II), both the microbiological and spectrophotometric data were in fair agreement, showing that approximately 80 per cent of the free purine was converted to desoxyriboside (microbiological assay, 74 per cent; spectrophotometric analysis, 85 per cent). Equilibrium data

(Paper II (5)) indicate that a maximum of 87 per cent conversion is the most that can be expected.

Experiments were carried out to test the possibility of forming desoxyribosides containing nitrogenous components other than the purines. This was done by incubating a series of compounds with desoxyribose-1-phosphate and rat liver nucleoside phosphorylase. Hypoxanthine and guanine were transformed to desoxyribosides as expected. Under the same conditions the following compounds produced no microbiologically active desoxyriboside when incubated with desoxyribose-1-phosphate: adenine,

TABLE II

*Synthesis of Hypoxanthine Desoxyriboside with Crystalline Cyclohexylamine Desoxyribose-1-phosphate**

25 μ l. of calf liver nucleoside phosphorylase (0.4 mg. of protein) were added to a mixture consisting of 2.5 μ M of cyclohexylamine desoxyribose-1-phosphate (1.03 mg.) and 2.5 μ M of hypoxanthine in 400 μ l. of 0.1 M tris(hydroxymethyl)amino-methane-HCl buffer, pH 7.4; temperature, 23.5°. Aliquots of 50 μ l. each were taken at intervals as indicated, and the enzymatic reaction stopped by heating for 2 minutes at 100°. Other conditions are mentioned in the table.

Experiment No.	Description	Desoxyriboside formed	
		Microbiological assay	Spectrophotometric data
		μ M	μ M
1	0 time (before addition of enzyme)	0	
2	2 min. incubation		0.2
3	61 " "	1.9	2.1
4	75 " "	1.9	
5	25 μ l. enzyme alone	0	

* See Table II, Paper II (5).

xanthine, thymine, uracil, uric acid, allantoin, nicotinamide, pyridoxine, alloxan, xanthopterin, folic acid, and pterioic acid.

The lack of reaction with adenine, also found to be negative in the case of ribose-1-phosphate (6), seems to indicate that the conversion of adenine to desoxyriboside occurs by a different enzymatic pathway. This puzzling situation with respect to adenine has recently been discussed (7).

In accordance with the observation of Klein (8), recently corroborated by Manson and Lampen (9), that the pyrimidine nucleosides are split by a specific enzyme different from purine nucleoside phosphorylase, no synthesis of pyrimidine desoxyriboside could be detected when either thymine or uracil was incubated with desoxyribose-1-phosphate in the presence of nucleoside phosphorylase from rat liver.

SUMMARY

The enzymatic synthesis of desoxyribosides when hypoxanthine or guanine is incubated with desoxyribose-1-phosphate has been substantiated by microbiological assay with *Thermobacterium acidophilus* R26. Good agreement between microbiological and differential spectrophotometric estimations of enzymatically produced desoxyriboside has been obtained. When nucleoside phosphorylase from rat liver is used, the formation of desoxyriboside with desoxyribose-1-phosphate appears to be specific for hypoxanthine and guanine; no synthesis occurs with adenine and pyrimidines.

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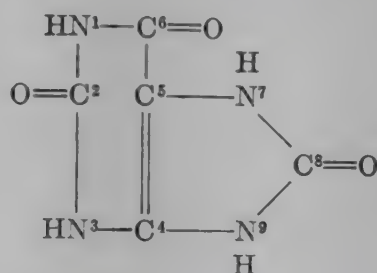
THE RÔLE OF SERINE AND ACETATE IN URIC ACID FORMATION*

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Recent investigations on the formation of uric acid have thrown considerable light on the nature of its precursors. In the pigeon the 4, 5, and 7 positions were shown to be derived from glycine, the 2 and 8 positions (the ureide carbon atoms) from formate or the carboxyl carbon of acetate, and position 6 from the respiratory CO₂ (1, 2).



In man the nitrogen in position 7 is derived from glycine (3). Similar mechanisms are involved in the formation of the nucleic acid guanine in yeast (4) and hypoxanthine in pigeon liver homogenates (5).

The earlier demonstration that L-serine is extensively converted to glycine (6) and, presumably, a 1 carbon intermediate provides ground for belief that the β -carbon atom of serine may be a precursor of the ureide carbon atoms of uric acid. Formate could enter these positions through condensation with glycine to form serine (7), which would act as a distributor or carrier of formate in the organism. In order to test this scheme serine labeled with N¹⁵ and with C¹⁴ in the β -carbon atom was synthesized and resolved. This report deals with the utilization of D- and L-serine for uric acid formation in the pigeon, and a reinvestigation of the utilization of acetate.

Positions 2, 7, and 8—A complete summary of the data obtained with

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† Life Insurance Medical Research Student Fellow, 1949–50. This report is from a dissertation to be submitted by David Elwyn in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

serine and acetate is presented in Table I. The utilization of the nitrogen of L-serine for nitrogen atom 7 of uric acid is more than twice as great as for the other nitrogen atoms. This is in accord with the known extensive conversion of serine to glycine (6), and the utilization of glycine for positions 4, 5, and 7 of uric acid (2, 3, 8). A part of the N¹⁵ in position 7 is

TABLE I

Utilization of β -Carbon and Nitrogen Atoms of Serine and Carboxyl Carbon of Acetate for Uric Acid Formation in Pigeons

Positions of atoms in uric acid	Activity or isotope concentration following administration of					
	DL-Serine*	L-Serine†	D-Serine†	1-C ¹⁴ - Acetate‡	1-C ¹³ - Acetate§	1-C ¹³ - Acetate§
	c.p.m.	c.p.m.	c.p.m.	c.p.m.	atom per cent excess	atom per cent excess
Total C	26,600	13,150	3170	17 $\pm$ 2	0.17	0.138
2 + 8	59,500	29,100	5980			0.050
8	60,700	29,100	5990	0 $\pm$ 7	0.029	0.048
4 + 5	4,250	3,490	2040	18 $\pm$ 8	0.17	0.150
4¶	1,490**	524**	679			
5	7,010	6,456	3401			
6	1,910***††	550**	648	49 $\pm$ 7	0.45	0.325
		atom per cent excess	atom per cent excess			
Total N		1.59	1.17			
1 + 3		1.19	1.24			
7		3.12	0.29			
9		0.86	1.91			

* 0.35 mm per 100 gm. The activity was 1.6×10^6 c.p.m. in the β -carbon atom.

† 0.59 and 0.66 mm per 100 gm. of L- and D-serine, respectively. The activity of the β -carbon atom was 3.13×10^6 c.p.m., and the amino group had 25.8 atom per cent excess N¹⁵.

‡ 0.20 mm per 100 gm. The activity of the carboxyl carbon was 46,500 c.p.m.

§ 0.72 and 0.44 mm per 100 gm. in the second and third acetate experiments, respectively. The carboxyl carbon was labeled with 61.7 atom per cent excess C¹³.

|| Calculated value. (The isotope concentration of nitrogen atom 9, being the difference between approximately equal quantities, is subject to a large error.)

¶ See foot-note 4 of the text.

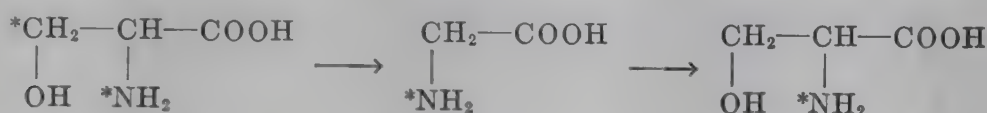
** Standard deviation between 5 and 10 per cent.

†† The activity of the respiratory CO₂ for the experimental period was 1380 c.p.m.

derived from the general nitrogen pool due to the removal of N¹⁵ from serine and glycine and resynthesis into glycine, the carbon atoms of which originated from other sources. Probably most of the isotope in position 7 following administration of D-serine is so derived, as indicated by an isotope concentration which is one-fourth of that found in the 1 and 3 positions. If a similar contribution by non-specific nitrogen sources is

assumed in the case of the L-serine experiments, then a correction of -0.30 ($1.19/4$) applied to the N^{15} value of the 7 position would give a more accurate estimate of the utilization of L-serine for position 7 via conversion to glycine. On this basis the N^{15} dilution in position 7 is $25.8/(3.1 - 0.3)$ or 9.2 .

The dilution of the C^{14} in the β -carbon atom of L-serine for the formation of the ureide carbon atoms of uric acid is 10.8 . This indicates that the β -carbon atom of L-serine is utilized as a source of these positions, either directly or following separation from the serine molecule, to about the same extent as serine nitrogen is introduced into position 7. The difference may be ascribed in part to the possibility of N^{15} -labeled glycine, derived from the administered serine, giving rise to serine containing N^{15} but lacking C^{14} in the β position.



This possibility is in accord with the rapid conversion of N^{15} -glycine to N^{15} -serine (9), and of α -labeled glycine to α,β -labeled serine (10, 11).

The utilization of the α -carbon atom of glycine for the synthesis of the ureide carbons of uric acid (8) can be explained by its conversion to α,β -labeled serine (10), and the subsequent entry of the β -carbon into the ureide positions of uric acid. Another possible pathway for the conversion of the α -carbon of glycine to the 2 and 8 positions of uric acid is via formation of doubly labeled acetate (12), of which the carboxyl carbon has been reported to be utilized in this reaction (1). It was, therefore, considered desirable to reinvestigate carboxyl-labeled acetate. From the last three experiments in Table I it can be seen that the utilization of the carboxyl carbon of acetate for the ureide carbon atoms is very small. The carboxyl carbon of acetate may, therefore, be removed from consideration as a precursor for the ureide carbon atoms. The participation of the α -carbon of glycine in the introduction of the ureide carbon atoms does not proceed through acetate.

The precursors discussed here have been administered in different doses and activities to animals of different body weights. In order to facilitate a comparison of the efficiencies with which various compounds are utilized for the synthesis of the same metabolic end-product, it is useful to calculate their coefficients of utilization (13).¹ This is done for a few of the

¹ The coefficient of utilization is derived from the isotope dilution formula (14), $b = a(x/y - 1)$, where a = millimoles of labeled compound administered per 100 gm. per day, b = millimoles of material per 100 gm. per day used to dilute a , x = isotope concentration of a , and y = isotope concentration of the compound isolated. The coefficient of utilization = $1000/b = 1000/a(x/y - 1)$. It is expressed as 1000 times the reciprocal of b so that it will show an increase with increasing utilization.

experiments reported here, as well as those of Karlsson and Barker (8) on α -labeled glycine and formate. The coefficients of utilization of formate and of the β -carbon of serine for the 2 and 8 positions are nearly the same. The rapidity of the conversion of serine to glycine is indicated by the utilization of serine nitrogen for position 7, which is approximately equal to that of the α -carbon of glycine for position 5.² The utilization of glycine α -carbon for the 2 and 8 positions is about one-third that of formate (8) and the β -carbon of serine. It would seem that of the compounds tested formate and serine (β -carbon atom) are the most immediate precursors of the 2 and 8 positions of uric acid. Which of these is the more immediate is difficult to determine in the intact animal.² However, the fact that they are almost equally utilized indicates that an interconversion of serine (β -carbon atom) and formate may exist which is more rapid than their incorporation into uric acid.³ It therefore appears that serine is a major source of formate as well as a product of formate metabolism (7, 16).

Positions 4 and 5—After the administration of either L- or D-serine there is an appreciable incorporation of β -carbon into the 4 and 5 positions.⁴ On the assumption that glycine is the precursor of these positions this would indicate a limited conversion of the β -carbon atom of serine to the α and carboxyl carbons of glycine.

When β -labeled serine and sodium benzoate were administered to rats,

² From experiments with the intact animal it is difficult to decide whether glycine or serine is the immediate precursor of the 4, 5, and 7 positions of uric acid. This is similar to the utilization of both glycine and serine for heme formation. In the latter case it was possible to determine that glycine was the immediate precursor by the use of a system in which incorporation of glycine into heme was much more rapid than the interconversion of glycine and serine (15). The generally accepted rôle of glycine in uric acid formation was assumed in this discussion.

³ This conclusion may be justified by a consideration of the general case when two labeled compounds, *A* and *B*, are administered to an animal and found to give rise to equal isotope concentrations in *C*. If it is assumed that *A* must proceed through *B* ($A \rightarrow B \rightarrow C$), then a rapid conversion of *A* to *B* is insufficient to explain the equal utilization of *A* and *B*, unless all of *A* is converted to *B*. If *A* undergoes reactions other than those giving rise to *B*, it is necessary that *B* should be converted rapidly to *A* as well as *A* to *B*. Unless this is so, the isotope concentration of *B* would be lower after administration of labeled *A* than after administration of labeled *B*. If the reversible reaction $A \rightleftharpoons B$ is much more rapid than the reaction $B \rightarrow C$, then the isotope level of *B* will be the same whether *A* or *B* is administered.

⁴ It was assumed that the carboxyl carbon of the isolated glycine was derived from position 4 of uric acid. The available evidence (17, 18) indicates that the nitrogen and α -carbon originate in positions 7 and 5, respectively. The carboxyl carbon, however, may be derived from position 4 or 6. In either case the value for position 5, obtained by difference, will remain the same. The unequivocal determination of the activity of position 4 is not essential for the discussion in this communication.

the excreted hippuric acid was labeled, three-fourths of the activity being in the α -carbon of the glycine.⁵ When it was fed to rats with γ -phenyl- α -aminobutyric acid, the activity of the excreted acetyl derivative showed an extensive conversion of serine to acetate, most of the label (85 per cent) being in the methyl group.⁵ Since pyruvate is probably the intermediate in this acetylation (13), this finding would be in agreement with the previously demonstrated deamination of serine to pyruvate *in vitro* (19).



Although the pyruvate involved in the acetylation was mostly β -labeled, a certain amount of randomization with the α -carbon had taken place,

TABLE II
Relative Efficiencies of Various Precursors for Uric Acid Formation

Positions of atoms in uric acid	Coefficients of utilization of					
	DL-Serine	L-Serine	D-Serine	1-C ¹³ - Acetate*	Formate†	2-C ¹⁴ - Glycine†
2 + 8	111	174	30	1.8	189	61
4	2.7	2.8	3.3	5.5†	1.3	56
5	13	36	17		5.0	193
6	3.5	3.0	3.1	12	0.9	8
7		207§	17			
1 + 3		82	77			

* Calculated from the last acetate experiment in Table I.

† Calculated from data kindly furnished by Dr. H. A. Barker which have been published in part (8). The weights of the pigeons have been estimated at 300 gm. for calculating the coefficients of utilization.

‡ Average value for 4 and 5 positions.

§ Calculated from the corrected dilution as discussed in the text.

as would be expected from the known participation of pyruvate in the Krebs cycle (20). A reversal of the deamination reaction would result in COOH, α , β -labeled serine which would give rise to COOH, α -labeled glycine.



The conversion of α -labeled pyruvate to α -labeled glycine (21), and the utilization of the α and carboxyl carbons of lactate for the 5 and 4 positions, respectively, of uric acid (2) have been interpreted in this manner.

⁵ Elwyn, D., and Sprinson, D. B., unpublished results.

If similar reactions of β -labeled L-serine are assumed to occur in the pigeon, the activity of the 4 and 5 positions is explained.

The β -carbon atom of D-serine is not utilized directly⁶ for positions 2 and 8, since the activity of positions 2 and 8 is only slightly higher than that of position 5. This is in agreement with the finding that D-serine is not a precursor of glycine (6). The distribution of activity in the uric acid is what would be expected if β -labeled D-serine gave rise to pyruvate and then to COOH, α, β -labeled L-serine.

Position 6—The activity of the respiratory CO₂ was determined only in the experiment with DL-serine, and found to be close to that of position 6. This is in agreement with the view that this position is formed by CO₂ fixation (2). From the coefficients of utilization for position 6 shown in Table II it can be observed that the oxidation of the β -carbon of serine in the pigeon is relatively slow.

EXPERIMENTAL

Synthesis of Labeled Compounds

C¹⁴-Formaldehyde—Labeled methanol was oxidized catalytically to formaldehyde (23). To prepare the catalyst (24) solutions of ammonium vanadyl oxalate (from 6.2 gm. of ammonium metavanadate) and ammonium molybdyl malate (from 4.4 gm. of ammonium molybdate) were poured on 60 gm. of grade RA-98 alundum chips 4 to 8 mesh.⁷ The mixture was evaporated, with stirring, until the chips were evenly coated with the sticky, black residue. Glass tubing of 10 mm. outside diameter was packed with this material to a length of 10 cm. and, while air was passed through the tube, heated gradually to 400° in an electric furnace. The catalyst was now ready for use (see Fig. 1).

Clean, dry air at room temperature was passed over the surface of 3 gm. of methanol, maintained at 34° in a water bath, at the rate of 810 ml. per minute. The resulting mixture was heated at 320° and passed over the catalyst at the same temperature. Under these conditions it was found by calibration of the particular apparatus used that this mixture contained 13 parts of air and 1 of methanol by weight, and had a time of contact with the catalyst of 0.12 second. Formaldehyde was collected in 5 ml. of water in two traps in series, in a yield of 65 to 70 per cent. After neutralization it was used immediately for the synthesis of serine.

⁶ An insignificant fraction of the activity in the 2 and 8 positions could have resulted from L-serine derived from the deamination of D-serine to hydroxypyruvic acid (22) and its reamination.

⁷ Kindly donated by the Norton Company, Worcester, Massachusetts.

3-C¹⁴-DL-Serine—Ethyl acetamidomalonate was condensed with the labeled formaldehyde (25) and DL-serine obtained in a 72 per cent yield.

$C_3H_7O_3N$. Calculated, N 13.3; found, N 13.6

Prior to injection this material was recrystallized from water and found to have an activity corresponding to 1.6×10^6 c.p.m. in the β -carbon atom.

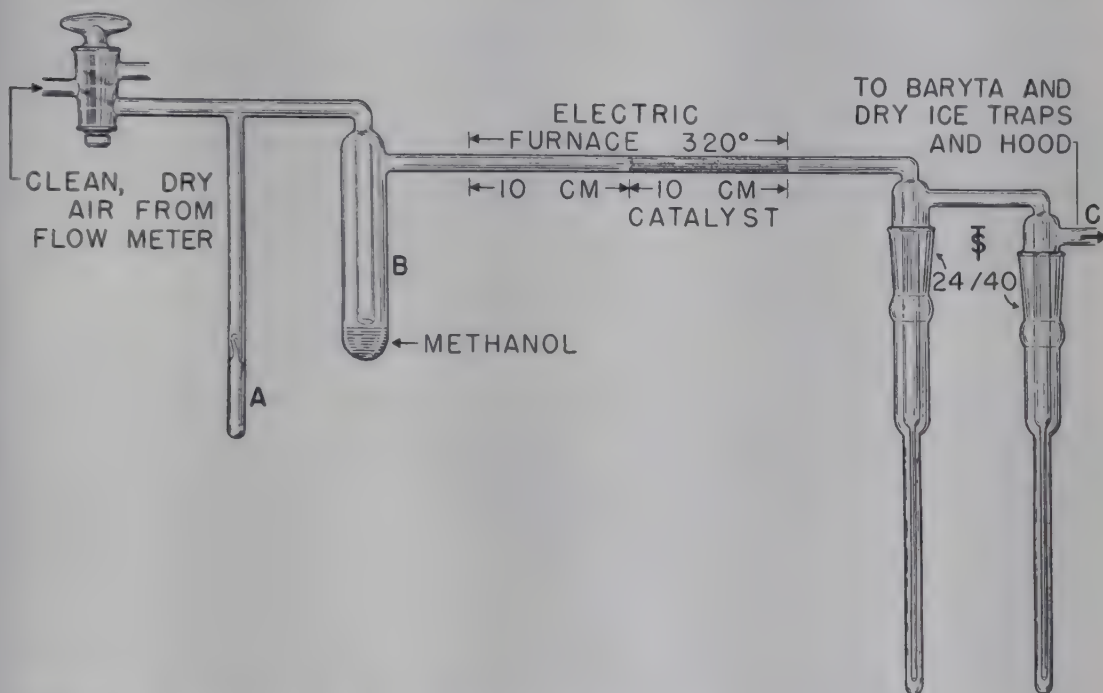


FIG. 1. Apparatus for the oxidation of methanol to formaldehyde. The tube containing labeled methanol in *A* and normal methanol above the break-off seal was fused to the apparatus. Mixing was procured by breaking the seal in the usual manner. The diluted methanol was distilled into trap *B* by applying suction at *C* and surrounding *B* with an acetone-dry ice mixture. For further details see the text.

*2,3-Deuterio-N¹⁵-DL-Serine*⁸—Glycine, prepared from N^{15} -potassium phthalimide (26), was benzoylated (27) in 95 per cent yield, and esterified in absolute ethanol with the aid of dry HCl. The yield, following recrystallization from ether-petroleum ether, was 96 per cent. The ethyl hippurate was condensed with 4 equivalents of dry ethyl formate in the presence of 4 equivalents of sodium in absolute ethanol (28, 29). In order to replace exchangeable hydrogen atoms with deuterium, the ether solution of ethyl formyl hippurate was dried over Na_2SO_4 and shaken with several portions of 99 per cent D_2O . It was then reduced with Al-Hg (28) in the presence of additional 99 per cent D_2O , filtered, the residue

⁸ The serine was labeled with deuterium in connection with another investigation.

extracted several times with ether and boiling benzene, and the combined ether and benzene solutions taken to dryness. Hydrolysis of the crude product with 20 per cent HCl, removal of benzoic acid (by ether extraction) and HCl (by distillation followed by exchange on Duolite-A4), and crystallization from a small volume of water with ethanol afforded DL-serine in a yield of 28 per cent based on glycine.

2,3-Deuterio-3-C¹⁴, N¹⁵-L-Serine and D-Serine—A mixture of 3-C¹⁴-DL-serine and N¹⁵-DL-serine was resolved by the method of Fischer and Jacobs (30). The yields of L- and D-serine were 36 and 43 per cent, respectively.⁹ These products were recrystallized once from water and had an activity corresponding to 3.13×10^5 c.p.m. in the β -carbon atom, 25.8 atom per cent excess N¹⁵, and 20.7 atom per cent excess deuterium.

C₃H₇O₃N (106.8, corrected for isotope content)

Calculated. N 13.4 (corrected for isotope content)

Found. L-Serine, N 13.4, $[\alpha]_D^{25} = -7.3^\circ \pm 0.2^\circ$ (7.94 % in water)

D-Serine, " 13.6, $[\alpha]_D^{27} = +7.4^\circ \pm 0.2^\circ$ (8.33 % " ")

1-C¹³-Sodium Acetate—Labeled KCN was treated with methyl sulfate in the presence of a small amount of alkali and the resulting acetonitrile distilled and hydrolyzed with 2.5 N NaOH (32). Following acidification with H₂SO₄ the acetic acid was distilled over from dilute solution and neutralized with NaOH, and the sodium acetate taken down to dryness. It had 61.7 atom per cent excess C¹³ in the carboxyl group.

1-C¹⁴-Sodium Acetate—Methyl magnesium bromide was carbonated with C¹⁴O₂. The carboxyl carbon had an activity of 46,500 c.p.m.

Isotope Analyses

Radioactivity Measurements—C¹⁴ activity was measured with a thin window Geiger-Müller counter either on the organic compounds isolated, or, when CO₂ was isolated, on BaCO₃. In the latter case the results were divided by 1.2 to correct for back-scattering.¹⁰ The counting vessels were sintered glass filter dishes of 2.41 sq. cm. area. When necessary, corrections to infinite thickness were made from known data (33). Except where otherwise indicated sufficient counts were taken to give a standard deviation of less than 5 per cent.

Mass Spectrometric Analyses—Samples of nitrogen were isolated as ammonia or the compound converted to ammonia by Kjeldahl digestion. Samples of carbon were isolated as CO₂ or the compound converted to CO₂ by combustion at 800°, and the gas collected in baryta. The ensuing procedures were those previously described (34).

⁹ A similar procedure for the synthesis of 3-C¹⁴-L- and D-serine from labeled methanol has been published recently (31).

¹⁰ Radin, N. S., unpublished results.

Administration of Compounds

The pigeons were fasted overnight and during the succeeding experimental period. The labeled compounds were dissolved in 8 cc. of water and injected intraperitoneally in four equal doses at 2 hour intervals.

Isolation of Uric Acid

Excreta were collected for a period of 24 hours following the first injection. Uric acid, isolated by the usual procedures (35, 36), was recrystallized to constant isotope content by dissolving in dilute NaOH solution, treating with activated charcoal when necessary, and acidifying with HCl. Respiratory CO_2 was obtained by passing CO_2 -free air consecutively through a large desiccator in which the pigeon was placed and carbonate-free NaOH solutions.

Degradation Procedures

Previously published degradation schemes (2, 37, 38) were used, except that carbon atoms 4 and 5 were not obtained as glyoxylic acid, but, together with nitrogen atom 7, as glycine (3, 39).⁴ Following removal of ammonia by aeration of the alkaline solution the glycine was benzoylated, the resulting hippuric acid recrystallized to constant activity from water, and the average isotope content of carbon atoms 4 and 5 determined. The hippuric acid was hydrolyzed with 20 per cent HCl, benzoic acid and excess HCl were removed, and the hydrolysate treated with ninhydrin in the presence of citrate buffer of pH 2.5 in order to isolate carbon atom 4 as CO_2 .⁴ The reaction mixture was made strongly alkaline with 50 per cent NaOH and the ammonia representing nitrogen atom 7 aerated into dilute H_2SO_4 for N^{15} analysis.

SUMMARY

L- and D-serine labeled with C^{14} in the β -carbon atom, N^{15} in the amino group, and deuterium in the α and β positions were prepared.

Following administration to pigeons it was shown that the β -carbon atom of L-serine is utilized for the ureide carbon atoms of uric acid to about the same extent as is the nitrogen for position 7. The incorporation into the ureide positions is about the same as that of formate. The utilization of serine nitrogen for position 7 is close to that of the α -carbon of glycine for position 5. A limited incorporation of C^{14} into position 5 took place following the administration of either L- or D-serine. The significance of these findings to the metabolism of serine, glycine, and formate, and the biosynthesis of uric acid has been discussed.

The reported utilization of the carboxyl carbon of acetate for the formation of the ureide carbon atoms of uric acid could not be confirmed.

We are grateful to Mr. I. Sucher for the C^{13} and N^{15} analyses, and to Miss M. R. Rother for the microanalyses.

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THE RELATION OF FOLIC ACID TO THE METABOLISM OF SERINE*

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The β -carbon atom of L-serine (1) and formic acid (2, 3) were both shown to participate to about the same degree as precursors of the ureide carbons of uric acid. Of all carbon sources investigated these two were used to the highest extent. L-Serine is also the most efficient known source of glycine in hippuric acid formation (4) and heme synthesis (5), as well as for the 4, 5, and 7 positions of uric acid (1). The reaction leading to the utilization of serine via glycine and a 1-carbon fragment is part of a cyclic or reversible process, since glycine can condense with formate, methyl groups of choline, and the α -carbon of glycine itself to give serine (6-9). A possible relation between the metabolism of the β -carbon atom of serine and the function of folic acid is suggested by the finding that the *Streptococcus lactis* R factor (rhizopterin) has the structure of N^{10} -formyl-pterioic acid (10), and that both the SLR factor and the N^{10} -formyl derivative of folic acid show folic acid activity for certain organisms (11, 12).

In order to determine whether such a relationship exists, L-serine, labeled with N^{15} , and sodium benzoate were administered to folic acid-deficient and control rats. Hippuric acid was isolated and its N^{15} concentration determined. The results (Table I) show that the dilution¹ of N^{15} was 3 times as great in the deficient as in the control rats.² This could be interpreted to mean (1) that the rate of conversion of serine to glycine is reduced in the deficient rats, or (2) that the amount of glycine available from endogenous sources, in these animals, is greater than normal.

The latter possibility was investigated by the same technique with N^{15} -labeled glycine in place of serine. From the results shown in Table

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† Life Insurance Medical Research Student Fellow, 1949-50. This report is from a dissertation to be submitted by David Elwyn in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

¹ Since the amounts of material fed per 100 gm. were nearly the same in all cases, comparison of isotope dilutions is justified.

² Similar experiments with α, β -deuterio-L-serine gave essentially the same results.

It can be seen that the administered glycine is diluted only one-half as much in the deficient as in the normal rats. This would indicate that in the deficient rats the production of glycine proceeds at one-half its normal rate, and that the greater isotope dilution obtained with serine in these animals is not due to an increase in the endogenous glycine.

TABLE I

Utilization of N¹⁵-Labeled L-Serine and Glycine for Hippuric Acid Formation in Normal and Folic Acid-Deficient Rats

The serine injected contained 25.8 atom per cent excess N¹⁵; the glycine, 8.42 atom per cent excess N¹⁵.

Diet	Na benzoate per 100 gm.	Amino acid per 100 gm.	N ¹⁵ in hippuric acid	Dilution*
Serine				
	mM	mM	atom per cent excess	
Normal†.....	0.50	0.15	1.600	16.2
Deficient.....	0.48	0.14	0.569	45.5
“.....	0.48	0.14	0.462	56.0
Glycine				
Normal.....	0.53	0.16	0.949	8.88
“.....	0.50	0.15	0.786	10.71
Deficient.....	0.61	0.18	1.703	4.94
“.....	0.69	0.21	2.080	4.04

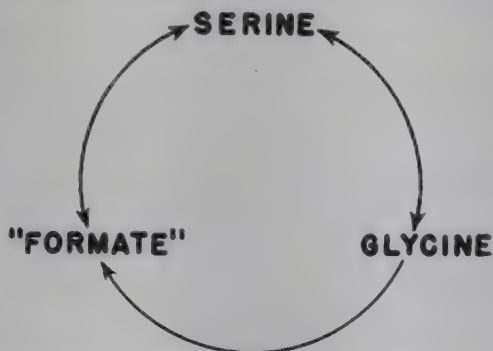
* Dilution = $\frac{\text{atom per cent excess of compound injected}}{\text{atom per cent excess of compound isolated}}$

† The urine of two rats was combined.

These results suggest that the increased dilution of the N¹⁵ in the hippuric acid of the deficient rats, following administration of isotopic serine, is due to a diminution of the rate of conversion of serine to glycine, which is about one-sixth of the normal value. The reverse of this reaction, *i.e.*, the condensation of glycine and formate to serine (6), is also related to the function of folic acid. A marked lowering of the utilization of C¹⁴-formate for the β -carbon atom of serine in folic acid-deficient rats has recently been reported (13).

The symptoms arising from glycine deficiency induced by feeding rats a diet high in benzoic acid were alleviated by folic acid (14). It has also been shown that the toxemia resulting from feeding a diet high in glycine can be cured or prevented by administration of folic acid (15). The hypothesis that folic acid is required for the reversible conversion of glycine to serine can account for both of these effects.

The mechanism for the rôle of folic acid in the interconversion of serine and glycine may involve the reversible formylation of the secondary amino group and be analogous to the introduction of an aldehyde group into certain aromatic compounds (16) by *N*-methylformanilide. The irreversible reaction of glycine to give "formate" (6, 8, 9), in conjunction with the reversible conversion of serine to glycine, affords an example of a metabolic cycle (17), as shown in the accompanying diagram. If the



assumption is made that both serine and "formate" can combine with folic acid, then the two products would be interconvertible by a loss or gain of a glycine molecule.

EXPERIMENTAL

Glycine labeled with N^{15} was prepared from isotopic potassium phthalimide (18). The N^{15} -labeled L-serine was prepared as previously described (1).

Adult, male, white rats were placed on a folic acid-free diet containing 1 per cent sulfasuxidine (19) to which were added 20 gm. of "methylfolic acid"³ per kilo. After 4 weeks they showed the loss of weight and hematological symptoms typical of folic acid deficiency (19). Control animals were maintained on the same diet, except that methylfolic acid was omitted and 10 mg. of folic acid per kilo of diet were added. Test substances with sodium benzoate were administered intraperitoneally in one dose when the deficient rats showed a definite decline in weight and an abnormal blood picture. The animals were fasted during the experiment.

The 24 hour urine specimens were acidified and extracted continuously with ether for 8 hours. The ether extract was evaporated to dryness and the crude hippuric acid recrystallized twice from water with use of activated charcoal when necessary. The melting points ranged from 187–190°. Analyses for N^{15} were carried out as previously described (20).

³ We are indebted to Dr. E. L. R. Stokstad for an ample supply of folic and methylfolic acids. Methylfolic acid is the crude product of the condensation of α,β -dibromobutyraldehyde, 2,4,5-triamino-6-hydroxypyrimidine, and *p*-aminobenzoyl-L-glutamic acid (19). From the method of synthesis the position of the methyl group is indefinite.

SUMMARY

The utilization of N¹⁵-L-serine and glycine for hippuric acid formation has been studied in normal and folic acid-deficient rats. From the lower utilization of serine and the greater utilization of glycine in the deficient rats it was estimated that in folic acid deficiency the rate of conversion of serine to glycine is reduced to one-sixth of the normal value. A possible mechanism for the rôle of folic acid in the metabolism of serine is discussed.

We wish to thank Mr. I. Sucher for the N¹⁵ analyses.

Addendum—Similar experiments have been performed in which one group of rats was maintained on the deficient diet containing methylfolic acid for 2 weeks while the controls received the same diet with addition of 100 mg. of folic acid per kilo. Although there were no symptoms of folic acid deficiency in either group at this time, the rate of conversion of serine to glycine in the animals maintained on the deficient diet (as measured by the methods described in this paper) was reduced to one-third of the normal.

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THE NITROGENOUS METABOLISM OF THE EARTHWORM (LUMBRICUS TERRESTRIS)

II. ARGINASE AND UREA SYNTHESIS

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Negligible amounts of urea were excreted by fresh¹ earthworms (*Lumbricus terrestris*); however, on fasting, the quantity of urea in the excreta increased notably (1). The oral administration of arginine was followed by an increased elimination of urea. The excretion of urea was also augmented, to a lesser extent, by the ingestion of citrulline with certain other amino acids. The data presented here were obtained in an attempt to clarify the mechanism of urea synthesis in the earthworm by studies *in vitro*. Arginase has been found to occur in the intestinal tissue of the earthworm and to be present in increased amounts in fasted animals.

EXPERIMENTAL

Animals—Mature earthworms, ranging in weight from 3 to 5 gm. each, were collected from the soil of the University campus and used for study within 24 hours after collection.

Phosphate Homogenates of Whole Worms—The Sørensen phosphate buffer (pH 7.5) was added to 40 gm. of worms to give a total volume of 100 ml. and the mixture was cooled and homogenized in a Waring blender. The homogenate was centrifuged briefly and the supernatant fluid was used as the enzyme preparation.

Phosphate Homogenates of Intestines—After the worms had been narcotized with gaseous carbon dioxide (1), the section of the gastrointestinal tract which contained the chlorogogue tissue was excised and washed with saline solution.² 2 ml. of the phosphate buffer (pH 7.5) were added for each gm. of tissue, and the mixture was homogenized in the apparatus of Potter and Elvehjem (3). The homogenate was diluted with the phosphate buffer to give a final concentration of 5 to 15 per cent of tissue, wet

¹ The term "fresh" as used in this discussion refers to worms which had been collected within 24 hours prior to the experimental procedures.

² The term "saline" is used to designate a 0.16 osmolar (0.08 N) solution of sodium chloride. This solution has an osmotic pressure approximately equivalent to that of the coelomic fluid of the earthworm (2).

weight. Aliquots of these homogenates contained approximately 0.059 mg. of nitrogen per mg. of dry tissue.

Incubation—3 ml. aliquots of the homogenized tissue, warmed to 29°, were added to 3 ml. of the desired substrate in a test-tube (24 × 200 mm.). After careful mixture, the solutions were adjusted to pH 7.5, and the incubation was begun. The concentrations of the substrate are indicated in the text. The incubation was carried out in a water bath at 29° for 1 hour; the tubes were shaken (115 oscillations per minute) during the incubation.

When a solution of cobaltous chloride was added to the medium, it replaced an equivalent quantity of water.

Procedure after Incubation—At the completion of an incubation period, the enzymatic activity was stopped by the addition of 1 drop of 10 per cent hydrochloric acid. The mixture was then heated for 2 minutes in a boiling water bath and was centrifuged. Aliquots of the supernatant fluid were adjusted to pH 6.4 by a solution of potassium hydroxide; bromocresol purple was employed as an internal indicator. 1 ml. aliquots were used for the determination of urea (1); all analyses were done in duplicate.

Substrates—The preparations of arginine and hydantoin were those used in work reported in our earlier paper (1). Glycocyamine was prepared by the method of Wheeler and Merriam (4) and hydantoic acid by the procedure of West (5).

Heidermanns had reported (6) that after incubation of the intact intestine of the earthworm with peptone there was an increased formation of urea. Since a similar incubation with arginine did not effect a synthesis of urea, the absence of arginase was assumed. We have confirmed these results with arginine by the use of intact intestine of fresh earthworms. However, we have not been able to demonstrate an increased synthesis of urea after incubation with Witte's peptone. We are unable to explain the discrepancy between our observations and those of Heidermanns.

It was possible, however, to demonstrate the presence of arginase in phosphate homogenates of the whole worm. In further studies, the intestinal tissue was removed and the arginase activities of the homogenates of the intestine and of the carcass (the tissue remaining after the removal of the intestine) were determined (Table I). Practically all of the arginase activity was localized in the wall of the intestine; the slight activity of the remaining tissues was probably due to small amounts of intestinal tissue not removed. No urease activity could be demonstrated with phosphate homogenates of whole worm.

Under our conditions of incubation, the optimal pH for arginase activity was found to lie between 7.2 and 8.6. The addition of Co^{++} in a concen-

tration of 10^{-3} M increased the activity of the arginase. The extent of this increase in activity was dependent, to some degree, upon the length of time the enzyme was allowed to remain in contact with the Co^{++} before the substrate was added. For example, the relative activities of the arginase in homogenates of intestine from fresh worms when measured without the addition of cobalt ions, with Co^{++} at a concentration of 10^{-3} M, and when the same concentration of Co^{++} was allowed to remain in contact with the enzyme for 30 minutes at room temperature before the substrate was added, were, respectively, 1.0, 1.3, and 2.1. When the Co^{++} was allowed to remain in contact with the enzyme for 3 hours before the addition of the substrate, the relative activity rose only to approxi-

TABLE I

Localization of Arginase in Intestine of Fasted Earthworms

Final concentration of arginine, 200 mg. per cent; of Co^{++} , 10^{-4} M. The values are calculated as micrograms of urea N formed per 100 mg. of tissue (wet weight) in 1 hour.

Experiment No.	Incubation mixture	Arginase activity
		γ
1	Intestine + arginine	356
2	" + "	364
3	Carcass + arginine	13
4	" + "	13
5	Intestine + water	5
6	" + "	5
7	Carcass + water	4
8	" + "	4

mately 2.2. In the absence of cobalt ions, the arginase activity of homogenates of the intestinal tract decreased rapidly on standing at room temperature. These observations are similar to those of Hunter and Downs (7), who studied the arginase of beef liver.

The arginase of the intestine of fresh worms (homogenates) failed to form urea from guanidoacetic acid (glycocyamine), hydantoin, or hydantoic acid.

In a series of experiments, preformed urea was determined in homogenates of the whole worm as fasting progressed up to 14 days. The urea content of the worms was calculated as micrograms of urea nitrogen per 100 mg. of whole worm (Series 1, Table II). Arginase activity was also measured in the homogenates of this series, the activity being calculated as micrograms of urea formed per 100 mg. of worm per hour. In another series (Series 2, Table II), arginase activity of intestinal homogenates was

determined and the results are expressed as micrograms of urea nitrogen formed per mg. of tissue nitrogen per hour.

For the determination of the urea content of the tissues, aliquots of the phosphate homogenates of the whole worm were heated, immediately after preparation, in a boiling water bath for 2 minutes, the solutions were centrifuged, and the centrifugates were analyzed for urea. In the determinations of arginase activity the incubation procedure was used with a

TABLE II

Urea Content and Arginase Activity of Homogenates of Intestines and Whole Worms

See the text for the details of procedure and calculation of results. The basis of calculation of arginase activity is different in the two series.

Series No.	Experiment No.	Condition of animal	Pre-formed urea N	Arginase activity
1. Whole animal	1	Fresh	γ	γ
	2	"	1	11
	3	"	1	11
	4	"	1	12
	5	Fasted 7 days	7	16
	6	" 7 "	10	19
	7	" 7 "	9	15
	8	" 14 "	52	36
	9	" 14 "	57	33
2. Intestine	10	" 14 "	60	41
	11	Fresh		26
	12	"		54
	13	"		62
	14	Fasted 29 days		504
	15	" 29 "		620
	16	" 29 "		600
	17	" 37 "		594
		" 37 "		618

final arginine concentration of 500 mg. per cent. No Co^{++} was added to homogenates of whole worm; in the studies with the intestinal homogenates, Co^{++} was added to activate the enzyme as already described. As shown in Table II, not only was there a progressive increase in the urea nitrogen of the whole organism during fasting, but there also occurred a parallel increase in arginase activity of homogenates of both the whole worm and the intestine. The increased arginase activity of homogenates prepared from the intestine of fasted worms compared with those from fresh worms could be demonstrated in the absence of Co^{++} , in the presence of Co^{++} (10^{-3} M), and also when Co^{++} was allowed to activate the enzyme

for periods up to 2.5 hours before the activity of the arginase was measured.

DISCUSSION

It has been shown in a previous paper (1) that the pattern of nitrogenous excretion in earthworms underwent a marked change during prolonged fasting, a change characterized by a progressive increase in the quantity of urea synthesized. The data here presented demonstrate that after 14 days of inanition the urea nitrogen content of the tissues had increased from negligible amounts to 57 mg. per 100 gm.

The presence of the enzyme arginase has been demonstrated in the intestinal tissue of both fresh and fasted earthworms. The arginase activity of the intestinal tract of worms after 4 weeks of inanition was approximately 10 times that of fresh worms. In other experiments it was observed that under conditions of incubation in which the intact intestine of *fresh* worms showed negligible arginase activity intact preparations of the intestine of *fasting* animals showed significant arginase activity. It may be noted that a 2-fold increase in arginase activity of the livers of normal rats either after a prolonged period of fasting or after the ingestion of a high protein diet has been reported (8).

Urea may be formed from uric acid by the activity of the enzymes uricase, allantoinase, and allantoicase (9) or the synthesis may involve the ornithine-citrulline-arginine cycle (10). Since neither uricase, allantoinase, nor allantoicase is stated to be present in the tissue of the earthworm (11, 12), the first mode of synthesis of urea is precluded. An essential factor in the ornithine cycle of Krebs and Henseleit is the enzyme arginase. The presence of this enzyme in tissues of the earthworm and the increased activity during fasting may indicate that the ornithine cycle functions in urea synthesis by the earthworm.

SUMMARY

1. The urea content of the earthworm increased markedly during inanition.
2. Arginase was present in homogenates of the intestinal tissue of the earthworms.
3. The arginase activity of homogenates of the intestine of the earthworm was increased about 10-fold after prolonged inanition (24 to 30 days).

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THE EFFECTIVENESS OF LACTIC ACID AS A HEMOLYTIC AGENT IN THE DETERMINATION OF BLOOD OXYGEN*

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The widely used manometric method of Van Slyke and Neill (1) for determination of the O_2 content or capacity of blood depends upon hemolysis of the erythrocytes with formation of an extracellular solution of HbO_2 and Hb, and upon liberation of O_2 by conversion of HbO_2 to MHb by the addition of ferricyanide. Although hemolysis of the erythrocytes results from the use of water alone, saponin is usually added to accelerate the process.

Van Slyke and Neill (1) and more recently Hiller, Plazin, and Van Slyke (2) have reported that commercial preparations of saponin vary markedly in their ability to hemolyze blood. The latter group found that a sample of Baker's saponin yielded only 53.9 per cent and Amend's saponin gave 83.6 per cent of the oxygen obtained when Kahlbaum's saponin was used as a standard. The errors that can arise in the manometric determination of O_2 in blood as a result of irregular and incomplete hemolysis are obvious.

Hiller *et al.* (2) suggested two substitutes for saponin. The first of these was 0.5 per cent infusion of either senega root, root soapwort, or quillaja soap bark, which yielded 99.6 to 100.5 per cent of the maximal quantity of O_2 present; however, this is not ideal since the infusion is crude and inconvenient to prepare. The second was 40 per cent urea-1 per cent egg albumin solution, which yielded 99.5 per cent of the maximal O_2 ; this involves an additional extraction period which increases by 25 per cent the time required for each determination.

This investigation was undertaken with the purpose of finding a more satisfactory hemolytic agent for use in the analysis of blood O_2 . As a result of systematic investigation of the constituents of the combined reagent employed to liberate blood O_2 and CO_2 by the technique of Van Slyke and Neill, it became apparent that, when lactic acid was combined with ferricyanide, maximal values for O_2 were obtained regularly, even in the absence of saponin or other hemolytic agents.

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Methods

The standard technique of Van Slyke and Neill (1) for the combined determination of O_2 and CO_2 upon 1.0 cc. of blood was used throughout except for omission or addition of reagents or alteration in extraction time. Human blood samples were used; their O_2 content varied from 13.4 to 23.20 volumes per cent. In all cases, *C* corrections were determined for the solutions used. Shaking speed was kept as constant as possible.

The amount of O_2 liberated by (a) lactic acid-ferricyanide, (b) lactic acid-saponin,¹ (c) lactic acid, (d) ferricyanide, (e) saponin, and (f) ferricyanide-saponin was determined and expressed as a per cent of that liberated by the combination of saponin-ferricyanide-lactic acid. The justifi-

TABLE I
Quantity of O_2 Liberated from Human Blood by Various Reagents

Reagent	No. of determinations	Per cent of maximal
Ferricyanide-saponin-lactic acid.....	42	100*
Ferricyanide-lactic acid.....	38	99.98†
Ferricyanide-saponin.....	15	78.2†
Saponin-lactic acid.....	2	88.5, 85.5
Lactic acid.....	2	83.4, 81.6
Saponin.....	2	64.0, 65.0
Ferricyanide.....	1	66

* The quantity liberated by this combined reagent was regarded as "maximal" (see the text).

† Average values.

fication for employing this last reagent as the standard is as follows: (a) the total O_2 did not increase with successive 3 minute periods of shaking at reduced pressure in the extraction chamber, and (b) spectrophotometric analysis for total hemoglobin agreed within 0.1 gm. with Hb determined manometrically as Hb capacity.²

Results

As is shown in Table I, the use of saponin-ferricyanide reagent yielded an average of 78.2 per cent of maximal values for O_2 . However, maximal values were obtained consistently by addition of ferricyanide and lactic acid without saponin. In thirty-eight comparisons of the effectiveness of ferricyanide-lactic acid with saponin-ferricyanide-lactic acid upon the same blood samples, the mean difference obtained was 0.004 volume

¹ Baker's saponin in a final concentration of 0.4 per cent was used.

² Determination by D. L. Drabkin.

per cent, with a standard deviation of the differences between reagents of ± 0.04 volume per cent.

Comparison was also made of the *rate* of liberation of O_2 by these two reagents. Fig. 1 shows that a 3 minute period was sufficient for each reagent to yield maximal values. Therefore the addition of saponin to ferricyanide-lactic acid neither increased the yield of O_2 nor accelerated its liberation.

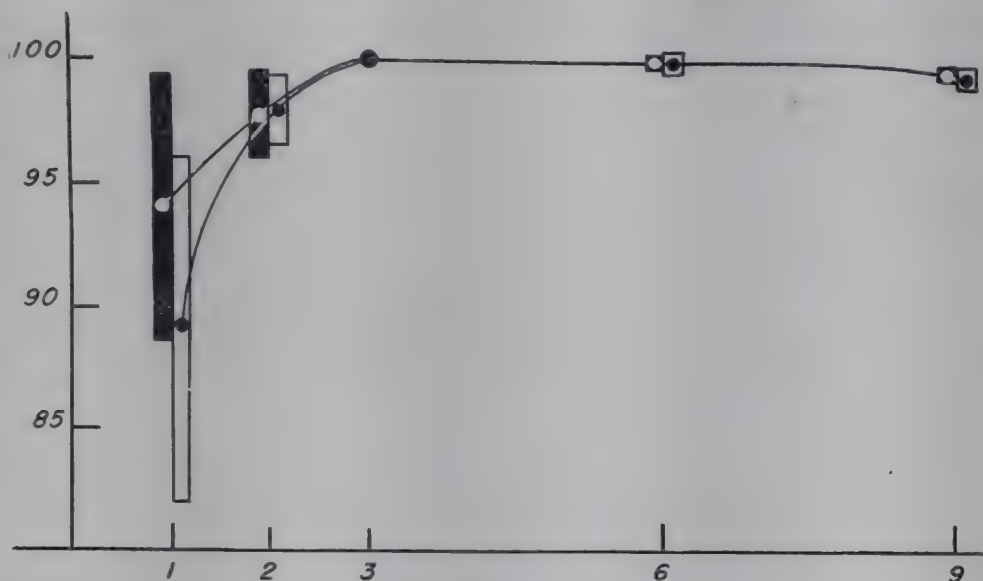


FIG. 1. Comparison of rate of oxygen liberation with lactic acid-ferricyanide and lactic acid-saponin-ferricyanide. Ordinate, per cent of maximal oxygen obtained; abscissa, extraction time in minutes. The black bar shows 1 standard deviation of the differences from the mean for saponin-lactic acid-ferricyanide. The clear bar shows 1 standard deviation of the differences from the mean for lactic acid-ferricyanide.

DISCUSSION

The liberation of O_2 from hemoglobin requires two steps: (1) hemolysis and (2) freeing of O_2 from HbO_2 . In the technique of Van Slyke and Neill, hemolysis is promoted by (a) hypotonicity, (b) the addition of saponin, (c) the presence of lactic acid, and (d) mechanical trauma, *i.e.* shaking with mercury. After hemolysis, the liberation of O_2 from HbO_2 is accomplished primarily by the conversion of HbO_2 to MHb (addition of potassium ferricyanide); it is favored also by exposure to low pO_2 ("vacuum" extraction), and by a shift in the HbO_2 dissociation curve (addition of lactic acid).

It is generally believed that saponin is the agent largely responsible for the first step. Our data indicate, however, that lactic acid is superior to saponin as a hemolytic agent, although the acid alone does not yield maximal values for oxygen because it does not complete the liberation of O_2 from HbO_2 . Ferricyanide alone does not give maximal values, for, although it converts Hb rapidly to MHb and quantitatively frees O_2 , it does not produce complete hemolysis. However, the combination of

ferricyanide and lactic acid (the latter in a final concentration of 0.4 per cent) regularly yielded maximal values for O_2 in the 3 minute extraction period whether saponin was present or not. This explains why incomplete hemolysis is not encountered when both O_2 and CO_2 are measured, since, in this procedure, lactic acid is added routinely to the ferricyanide-saponin reagent to insure liberation of CO_2 .

Although the effectiveness of lactic acid as a hemolytic agent has been overlooked previously, another acid, acetic acid, is employed routinely to hemolyze erythrocytes in the clinical enumeration of white blood cells. Both acids probably act by causing denaturation of proteins in the cell surface, thus permitting passage of cations across the erythrocytic membrane and consequent swelling of the cells (3). Saponin may be less satisfactory as a hemolytic reagent because it combines with plasma proteins as well as with proteins in the cell membrane, and also because it possesses an "antihemolytic" activity in certain concentrations owing to its coating the cell surface.

Although saponin appears to be superfluous if lactic acid is added,³ we have continued to add saponin to the ferricyanide-lactic acid reagent because saponin reduces the soiling of the chamber and forms a more complete emulsion with the caprylic alcohol which is routinely used as a defoaming agent. However, we do not recommend that saponin-ferricyanide be employed in the determination of blood O_2 unless lactic acid is also added.

SUMMARY

1. Reagents used in the Van Slyke-Neill manometric technique for measuring oxygen and carbon dioxide in blood were tested singly and in combination for their ability to yield maximal values for blood O_2 .

2. The use of saponin-ferricyanide resulted in low and inconsistent values. The combination of lactic acid with ferricyanide alone, or with saponin-ferricyanide, yielded maximal values.

3. Difficulties in the measurement of blood oxygen caused by incomplete hemolytic action of saponin may be eliminated if the reagent employed in combined analysis of O_2 - CO_2 (lactic acid-ferricyanide-saponin) is also used when blood O_2 alone is being measured.

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³ In ten analyses in which CO_2 was determined, both with and without saponin, the difference between the means was 0.01 volume per cent and the standard deviation of the differences between values for the two reagents was ± 0.05 volume per cent.

METABOLISM OF THE THYROID HORMONE IN THE RAT AS SHOWN BY PHYSIOLOGICAL DOSES OF LABELED THYROXINE

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Since thyroxine, uncombined in peptide linkage, has been detected by isotope dilution and paper chromatography in thyroid tissue (1, 2) and in plasma (1-3), it is likely that this substance is the hormonal secretion of the thyroid gland. Previously, the fate of labeled thyroxine has been determined after the injection of large amounts (4-6), considerably in excess of the few micrograms which constitute the daily thyroxine secretion of the rat thyroid (7). In the present work, methods were devised by which it was possible to prepare 0.007 γ of labeled thyroxine containing enough radioactivity to be easily traced in the body of the rat. The distribution of thyroxine thus obtained was then found to be comparable to that of radioiodide (NaI^{131}) in rats sacrificed long enough after injection to insure the conversion of the radioiodide to thyroid hormone.

Methods

Chemical Synthesis of Labeled DL-Thyroxine—DL-Thyroxine labeled in the 3' and 5' positions was prepared from DL-diiodothyronine in the following manner. The radioactive iodine as iodide, mixed with 0.70 mg. of potassium iodide and a drop of strong ammonium hydroxide, was brought to a volume of about 0.1 cc. on a water bath under nitrogen. By the addition of 0.05 cc. of 1 N sulfuric acid and 0.05 cc. of potassium iodate solution containing 80 mg. per cc., elemental iodine was obtained.¹ To this mixture 0.68 mg. of DL-diiodothyronine, dissolved in 0.1 cc. of strong ammonium hydroxide, was added rapidly. 10 per cent acetic acid was added dropwise until a precipitate appeared and the iodine color returned. The precipitate was removed by centrifugation, redissolved in strong NH_4OH , and added to the supernatant containing free iodine for the iodination of any remaining diiodothyronine. This procedure was repeated three times. After the final acidification, 0.1 cc. of 0.1 N thiosulfate was

¹ In some batches of iodine, the amounts of bisulfite present interfered with the formation of free iodine. This was overcome by oxidation with hydrogen peroxide in an acid medium. The excess peroxide was removed by adding ammonium hydroxide and evaporating the mixture to dryness. Subsequent treatment was as described in the text.

added to remove the residual iodine. The precipitate was washed twice with 0.2 cc. of 1 per cent acetic acid, dissolved in 0.2 cc. of 2 N sodium hydroxide, and reprecipitated twice with a few drops of 10 per cent acetic acid. The final thyroxine precipitate was dissolved in a minimal amount of hot 0.1 N potassium carbonate; 0.05 cc. of 15 per cent sodium chloride was added, and, on cooling, the alkali salt precipitated. After centrifugation, the residue was dissolved in 0.05 cc. of 2 N sodium hydroxide and diluted to 5 cc.

The iodine content, estimated by the method of Groak (8), was found to be equivalent to 655 γ of thyroxine, while the radioactivity was equal to 32.6 per cent of the starting material, which had an activity of 5 mc. By means of the isotope dilution procedure detailed below, 95 per cent of the radioiodine was found to be in the form of thyroxine.

Extraction of Labeled L-Thyroxine from Plasma—Female albino rats, kept on Remington's iodine-deficient Diet 342 (9), supplemented with 10 per cent brewers' yeast, were injected subcutaneously with about 50 μ c. of carrier-free radioactive iodide. The animals were exsanguinated 48 hours later, and the heparinized plasma was extracted with twice its volume of *n*-butanol, and three times again with an equal volume of butanol. The combined butanol fractions were then evaporated *in vacuo* at room temperature. The dry residue was taken up in about 0.5 cc. of distilled water. An aliquot of this solution was removed for the determination of thyroxine content by means of isotope dilution (see below). If the radioactivity in this aliquot proved to consist of 83 per cent or more of radiothyroxine, the remainder was used for injection. On the average, the injection mixtures contained 92 per cent of their radioactivity in the form of thyroxine. Because of the mild extraction method, it seems likely that little or no racemization occurred, thus giving the L-amino acid labeled in any one of the four positions.

Isotope Dilution Technique—25 mg. of inactive DL-thyroxine were added to the sample of radioactive material, dissolved with about 3 drops of 2 N sodium hydroxide, and diluted to 5 cc. with water. An aliquot was removed for iodine estimation (8) and for measurement of radioactivity to determine the specific activity. 3 drops of 10 per cent acetic acid were added in order to precipitate thyroxine, which was then washed with two 0.5 cc. portions of distilled water and dissolved in a minimal amount of hot 0.1 N potassium carbonate (about 0.5 cc.). On cooling, the potassium salt of thyroxine separated and, after removal of the supernatant, was dissolved in 1 cc. of 0.2 N sodium hydroxide in 70 per cent ethanol. Thyroxine was precipitated with acetic acid, washed with water, redissolved in alkaline alcohol, and reprecipitated as the potassium salt four times. Aliquots were taken from the alcoholic solutions of the second, third, and fourth salts for the determination of specific activity, which became constant with

the formation of the second potassium salt. The percentage of the constant specific activity over the initial specific activity indicated the proportion of the radioactivity present as thyroxine in the solution injected.

Distribution of Radiothyroxine—Female albino rats, fed on an adequate iodine intake (Purina fox chow) and weighing 80 to 120 gm., were injected with radiothyroxine intravenously, via the femoral vein. After periods of 2, 24, or 72 hours, the animals were etherized and exsanguinated via the inferior vena cava. The organs and tissues were weighed wet and then put into solution in 2 N sodium hydroxide on a water bath for radioactivity measurement as previously described (5). In calculating the total plasma volume, the formula $\text{plasma volume} = 0.122(\text{body weight})^{0.778}$ was used (10).

Those samples containing a sufficient amount of radioactivity were fractionated with butanol into thyroxine-like and non-thyroxine iodine, with no further hydrolysis than had occurred in the course of the homogenization of the organs in sodium hydroxide. 50 γ of inactive DL-thyroxine were added to each sample. The material was then extracted with butanol (5). The radioactivity found in the butanol extract or thyroxine-like fraction was expressed as a percentage of the total activity; *i.e.*, the sum of the activities of the butanol and alkali fractions. In control runs, 88 per cent of added radiothyroxine was recovered by this procedure.

Three series of distribution experiments (Fig. 1, Tables II and III) were carried out. (1) Three groups of two animals were injected with 20 γ of DL-thyroxine synthesized from DL-diiodothyronine, and sacrificed at 2, 24, and 72 hours after injection, respectively. (2) Three groups of two animals were injected with about 0.07 γ of L-thyroxine extracted from rat plasma, and sacrificed at 2, 24, and 72 hours after injection, respectively. (3) Three single animals were injected with about 0.007 γ of L-thyroxine extracted from rat plasma, and sacrificed at 2, 24, and 72 hours after injection, respectively. The amounts of thyroxine injected in the latter two series were calculated from the iodine content of the butanol extract of the plasma of the iodine-deficient donor animals; *i.e.*, about 1.2 γ per 100 cc. of plasma.

Additional data on the *excretion* of thyroxine were obtained in an experiment in which three female albino rats, weighing about 170 gm., were injected intravenously with 0.001 γ of thyroxine (Table I).

Distribution of Radioiodide—For purposes of comparison, six female albino rats, fed on Purina fox chow and weighing 80 to 120 gm., received an intravenous injection of carrier-free radioiodide, and were sacrificed in groups of two after 2, 24, and 72 hours. The radioactivity of the organs of these animals was estimated and fractionated with the same methods that were used on the organs of the thyroxine-treated animals (Table IV).

Finally an attempt was made to identify thyroxine in the peripheral or-

gans of radioiodide-treated rats by *isotope dilution*, with use of animals on a low iodine diet, which were sacrificed 48 hours after the injection of about 50 $\mu\text{c.}$ of NaI^{131} . The organs of these animals were homogenized in a Waring blender with 10 cc. of 0.9 per cent sodium chloride per gm. of tissue. The mixture was extracted three times with butanol containing 10 γ

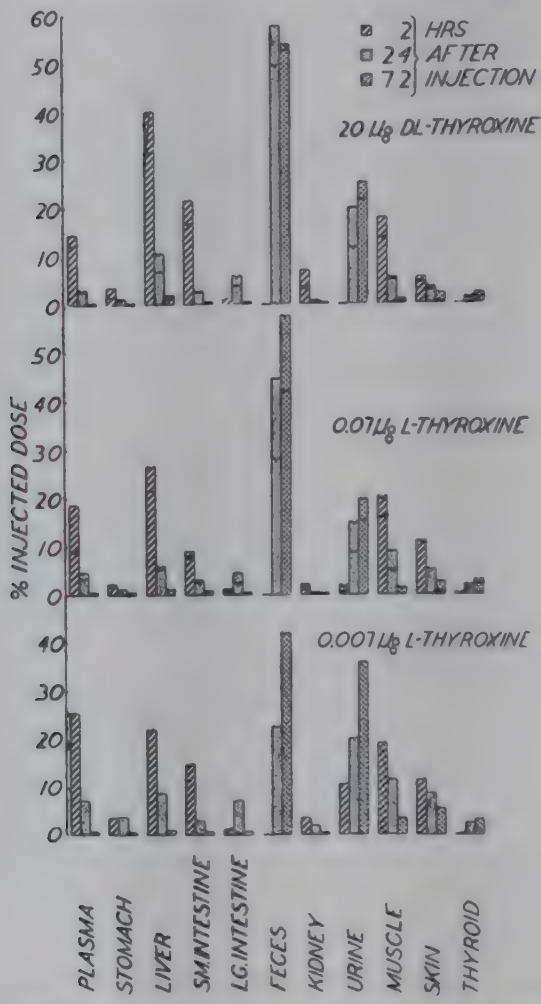


FIG. 1. Distribution of labeled thyroxine. The columns indicate the percentages of the injected doses present in the various organs at the three time intervals after injection. With the larger two doses, the values for the individual animals are indicated by the straight and notched lines, respectively, at the top of the column.

of carrier thyroxine per cc. The combined butanol fraction was evaporated under reduced pressure to about 5 cc. and washed twice with 5 cc. of 4 N sodium hydroxide containing 5 per cent sodium carbonate. Isotope dilution was then carried out on the dried butanol extract.

Results

Distribution of Radiothyroxine—The distribution of thyroxine on the basis of the percentage of the injected dose (Fig. 1) was qualitatively similar

for the three dose levels. Thus, as early as 2 hours after injection, over 70 per cent of the dose had left the circulation to find its way mainly into the liver, muscles, small intestine, and skin. At 24 and 72 hours, most of the injected radioactivity was found in feces and urine. With the exception of the thyroid gland, in which there was a slow increase in radioactivity with time, radioactivity in the organs gradually decreased. However, the large intestine showed a maximal value at 24 hours, while at the same time there was a marked decline in the radioactive content of the small intestine and, on the other hand, large amounts of radioactivity in the feces. Apparently, the material which entered the small intestine at 2 hours had passed into the large intestine and feces at the end of 24 hours.

A quantitative comparison of the three dose levels showed that with decreasing dosage there was an increase in the percentage of the dose remain-

TABLE I

Per Cent of Injected Dose Present in Feces and Urine at Various Time Intervals after Injection of 0.001 γ of Radiothyroxine

Body weight <i>gm.</i>		Amount excreted from		
		0-24 hrs.	24-48 hrs.	48-72 hrs.
183	Feces	30.7	15.8	4.1
	Urine	27.9	8.9	2.9
157	Feces	22.0	12.9	1.8
	Urine	26.4	14.1	2.4
164	Feces	33.4	18.8	2.1
	Urine	20.5	13.5	3.1

ing in the plasma, with a corresponding decrease in the proportion found in the liver. This phenomenon was most apparent 2 hours after injection (Fig. 1). At 24 hours and subsequently, the differences were most evident in the amounts of radioactivity found in the excreta, a lower fecal and a higher urinary excretion occurring with a decreasing dose at either time interval. This was borne out by the experiment with 0.001 γ of labeled thyroxine, in which about 40 per cent of the radioactivity passed into the urine and about 50 per cent into the feces (Table I).

The *concentrations* of the radioactivity in the various organs (Table II) showed the highest values in plasma, liver, and kidney at all time intervals, the highest plasma concentration being obtained with the smallest dose. Of the remaining organs, the lung, non-thyroid endocrine organs, cardiac muscle, pancreas, and salivary glands showed the higher concentrations. No remarkable concentration could be detected in the hypophysis, contrary to results obtained in the rabbit (4, 11).

TABLE II

Concentration of Radioactivity in Organs and Tissues of Rat after Injection of Labeled Thyroxine*

	20 γ DL-thyroxine			0.07 γ L-thyroxine			0.007 γ L-thyroxine		
	2 hrs.	24 hrs.	72 hrs.	2 hrs.	24 hrs.	72 hrs.	2 hrs.	24 hrs.	72 hrs.
Plasma	3.09	0.62		4.35	1.08	0.11	5.97	1.38	0.13
	3.57	0.69	0.08	2.18	0.80	0.13			
Red blood cells	0.29	0.07					0.19	0.03	—
	0.17	0.08	0.002						
Liver	6.95	1.27	0.28	4.16	0.97	0.19	5.00	1.71	0.23
	7.85	2.35	0.26	5.25	1.00	0.20			
Pancreas	0.59	0.16	0.01	0.80	0.33	0.04	1.29	0.49	0.10
	0.64	0.20	0.02	0.54	0.23	—			
Submaxillary and sublingual	0.70	0.15	0.01	0.79	0.29	0.03	1.55	0.20	0.22
	0.57	0.17	0.02	0.80	0.60	—			
Kidney	8.50	0.77	0.08	2.48	0.74	0.10	3.96	1.90	0.12
	4.09	0.69	0.05	1.78	0.65	0.16			
Adrenals	1.22	0.24	0.04	1.20	0.42	0.08	1.87	0.76	0.93
	1.05	0.24	0.03	0.90	0.33	—			
Ovaries	0.94	0.02	—	1.83	0.46	0.06	1.74	—	—
	0.69	0.03	—	0.72	0.41	—			
Hypophysis	1.02	0.27	—	1.13	—	—	—	—	—
	1.10	0.13	—	1.42	—	—			
Spleen	0.49	0.15	0.04	1.50	0.25	0.30	1.00	—	—
	0.48	0.25	0.10	1.21	0.27	—			
Lymph nodes	0.79	0.13	0.01	0.62	0.30	0.04	1.25	0.33	—
	0.73	0.21	0.02	0.54	0.26	0.03			
Thymus	0.32	0.08	0.009	0.11	0.12	0.02	0.54	—	—
	0.25	0.17	—	0.68	0.16	—			
Lungs	1.05	0.28	0.03	1.40	0.41	0.06	2.21	0.57	—
	1.24	0.44	0.02	1.15	0.35	0.06			
Skin	0.36	0.16	0.03	0.61	0.27	0.14	0.67	0.48	0.36
	0.27	0.19	0.01	0.29	0.29	0.06			
Skeletal muscle	0.38	0.10	0.02	0.36	0.18	0.03	0.40	0.22	0.07
	0.30	0.10	0.01	0.40	0.13	0.03			
Cardiac muscle	1.08	0.16	0.02	1.18	0.39	0.05	2.92	0.36	
	0.78	0.18	0.01	0.71	0.25	0.04			
Uterus	0.81	0.23	0.01	0.33	0.30	0.03	1.65	0.23	—
	1.17	0.21	0.02	0.60	0.24	0.04			
Brain	0.26	0.12	0.007	0.28	0.21	0.10	0.60	0.40	—
	0.27	0.08	—	0.18	0.17	—			

The values shown as dashes represent a counting rate below the level of statistical significance.

*Expressed as the ratio of the radioactivity per mg. of organ weight to the injected radioactivity per mg. of body weight.

Butanol fractionation of some of the samples (Table III) revealed that the thyroxine-like fraction was high at 2 hours in most of the organs and,

TABLE III

Fractionation of Organs and Excreta at Various Time Intervals after Injection of Various Doses of Labeled Thyroxine

The results are expressed as per cent total I^{131} present in the butanol fraction. All blank spaces correspond to samples with insufficient radioactivity for fractionation.

	20 γ DL-thyroxine			0.07 γ L-thyroxine			0.007 γ L-thyroxine		
	2 hrs.	24 hrs.	72 hrs.	2 hrs.	24 hrs.	72 hrs.	2 hrs.	24 hrs.	72 hrs.
Plasma	69	61	62	79	77	83	86	80	
	73	55	73	82	61	54			
Stomach	9	3	7	27	5	18	13		
	16	3	9	19	8	10			
Small intestine	63	39	38	25	31	20	74		
	54	42	39	67	39	82			
Large "	31	50	49		14	61		40	
	37	48	43		42	85			
Feces		46	50		12	54		27	18
		41	49		40	78			
Liver	70	52	17	85	74	44	79	50	
	71	58	20	86	70	32			
Kidney	64	55	39	83	69	64			
	65	52	32	90	71	34			
Urine	4	1	1	6	1	4	3	3	3
	2	1	1	7	10	20			
Spleen	69	41	28	82	61	46			
	56	38	28	90	54	59			
Lungs	60	64	38	86	69				
	59	41	43	72					
Skin	63	31	20	61	64	13			
	53	44	33	64	37	70			
Skeletal muscle	68	60	56	60		59			
	75	66	50	64	62	63			
Cardiac "	75	63	51						
	69	64	39						
Adrenal	61	51							
	55	49							

except for the plasma, decreased steadily with time. Two other exceptions to this pattern were the stomach and urine, in which very little of the radioactivity was present in the thyroxine-like form at any time interval. Note that the values in Table III are about 12 per cent too low (see "Methods").

Distribution of Tracer Radioiodide—The distribution pattern of radio-

active iodide differed from that of radiothyroxine for a short interval after injection. Thus, at 2 hours the radioactivity of the stomach was high (18 per cent of the injected dose) and that of the liver, low (1 per cent). Within 24 hours after administration, 60 to 80 per cent of the injected dose passed into the urine and somewhat less than 10 per cent into the feces. The entry of radioiodine into the thyroid was rapid, reaching a maximum (10 per cent) by 24 hours and declining later. In the organs at 24 and 72 hours, when presumably the thyroid had transformed radioiodine into its hormone, the distribution of the radioiodine was quite similar to that of thyroxine. Thus, of the total radioactivity present in the body at 24 hours, exclusive of the thyroid, the liver contained 10 per cent, the plasma 4 per cent, the stomach 4 per cent, the muscle 26 per cent, and the kidney about 1.5 per cent. Accordingly, the butanol-soluble radioactivity of these organs, which was low soon after injection, became high at the later time

TABLE IV

Fractionation of Organs at Various Time Intervals after Injection of Carrier-Free Radioiodide

	Per cent of I^{131} in butanol-soluble form at					
	2 hrs.		24 hrs.		72 hrs.	
Plasma.....	5.6	7.2	50.5	66.0	50.0	39.2
Liver.....	1.2	1.6	67.0	75.5	68.1	62.0
Muscle.....	1.2	1.0	36.8	34.4	34.0	20.0
Skin.....	0.7	0.7	6.3	8.9	2.4	2.4

intervals (Table IV). The only exception was the skin, which fixed as much as 37 per cent of the radioactivity of the body, but in a form insoluble in butanol.

By isotope dilution, thyroxine was demonstrated in the butanol fraction of various organs 48 hours after radioiodide injection. It constituted a large proportion, but not all, of the butanol-soluble iodine; *e.g.*, 81 per cent of the liver, 57 per cent of the kidney, and 49 per cent of the muscle butanol fractions respectively. It may be noted that, since there was no hydrolysis of the tissues preceding extraction in these samples, the thyroxine of the peripheral organs is not bound in peptide linkage.

DISCUSSION

The total plasma volume of the animals receiving the labeled thyroxine was calculated to contain 0.25 γ of thyroxine, since the iodine content of the butanol extract of plasma from Purina-fed animals was equal to about 4 γ per 100 cc. It was felt that the lowest doses of thyroxine used (0.007

and 0.001 γ) would not increase this amount significantly and, therefore should not interfere with normal physiological processes. Furthermore, since thyroxine combines easily with plasma proteins to form a complex which may be dissociated with butanol (2), as does the endogenous hormone (2, 3, 12), it was felt that the injected material would be similarly bound and would therefore behave like a tracer of the thyroid hormone. In favor of this assumption was the fact that the distribution of thyroxine was similar to that obtained 24 to 72 hours after the administration of radioiodide; that is, after a sufficient interval for the conversion of this iodide into thyroid hormone (see "Results" and Table IV). It was then concluded that the distribution of at least the smallest doses of labeled thyroxine reflected that of the endogenous hormone.

It is generally assumed that a hormone concentrates in its target organs. However, soon after injection, tracer doses of thyroxine were usually less concentrated in the organs than in plasma (Table II). There was a rapid fall of concentration with time in both, but the rate of fall was more rapid in the plasma than in the following organs: liver, kidney, skin, brain, spleen, muscle, fat, and perhaps adrenal and pancreas. This may indicate that the initial entry of thyroxine into the organs is dependent on the plasma level, but that the turnover of the thyroxine iodine is slower in the organs than in plasma.

The slower turnover of thyroxine in the organs was probably related to its conversion into butanol-insoluble metabolites, since most of the organs showed a steady decrease with time in the proportion of their butanol-soluble radioactivity, while in the plasma this fraction remained at about the same high level at all time intervals (Table III). The presence of thyroxine in the organs may be associated with biological activity, since this hormone was required by the thyroidectomized rat in order to maintain the normal weight of liver, kidney, spleen, and adrenal (13) as well as the normal histology of skin and muscle (14). The high concentration of thyroxine in the liver may be also related to the biliary secretion of thyroxine, since it has been shown previously that the fecal radioactivity came from this organ through the bile (5, 6).

There is a rapid fall in the thyroxine content of the whole body, as well as in that of the individual organs. The retained thyroxine, calculated from the difference between the radioactivity injected and excreted (Table I), showed a roughly linear relation to time. 50 per cent of the dose was thus found to be excreted in 18 to 24 hours (that is, the biological half life). Therefore, the time for the complete renewal of the body thyroxine of these animals ($1.44 \times$ the half life (15)) ranged from 25 to 35 hours.

The elimination of radioactivity from the body takes place in urine and mostly in feces (Fig. 2). However, the excretion was less pronounced with

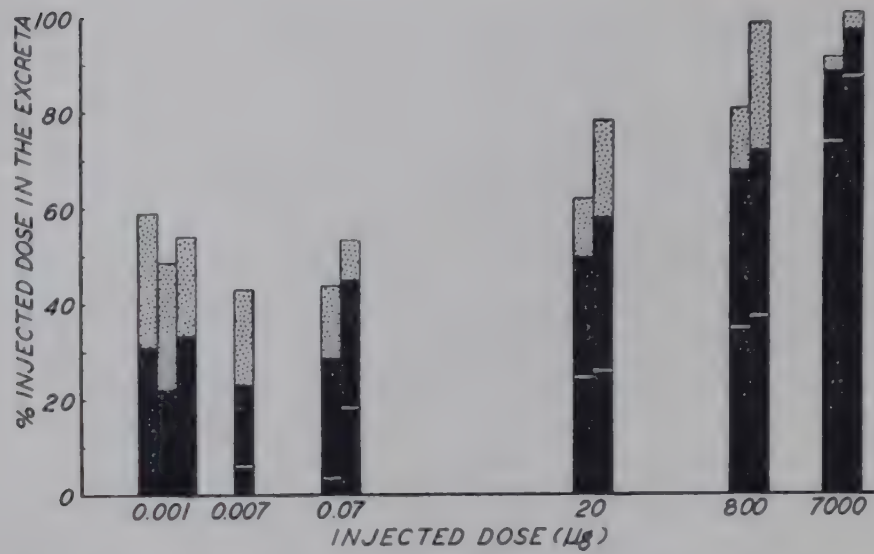


FIG. 2. A comparison of the excretion of radioactivity over the 24 hour period following the injection of various doses of radiothyroxine. The figure has been constructed from results presented in this paper and in a previous publication (5). The stippled portions of the columns represent the urinary I^{131} excretion; the solid black, the fecal I^{131} excretion. The distance between the base-line and the white lines indicates the amount of fecal radioactivity present in a butanol-soluble form. As the dose increases, both the percentage of the dose excreted in the feces and the proportion present in the butanol-soluble fraction increase.

TABLE V

Comparison of Average I^{131} Concentrations* of Radioactivity in Organs 2 Hours after Injection of Various Doses of Radiothyroxine

	Dose Injected				
	2000 γ†	800 γ†	20 γ	0.07 γ	0.007 γ
Blood plasma.....	0.38	0.94	3.33	3.26	5.97
Liver.....	2.67	2.78	7.40	4.70	5.00
Pancreas.....	0.71	0.48	0.61	0.67	1.29
Submaxillary gland.....	0.26	0.40	0.63	0.79	1.55
Kidney.....	1.08	1.54	6.29	2.13	3.96
Adrenals.....	0.50	0.57	1.13	1.05	1.87
Ovaries.....	1.02	0.41	0.81	1.27	1.74
Spleen.....	1.25	0.59	0.48	1.35	1.00
Lymph nodes.....	0.22	0.57	0.76	0.58	1.25
Thymus.....	0.10	0.19	0.28	0.39	0.54
Lungs.....	1.98	1.77	1.14	1.27	2.21
Skin.....	0.25	0.39	0.31	0.45	0.67
Skeletal muscle.....	0.20	0.27	0.34	0.38	0.40
Cardiac “.....	0.27	0.47	0.93	0.94	2.92
Uterus.....	0.19	0.51	0.99	0.46	1.65

* Expressed as the ratio of the radioactivity per mg. of organ weight to the injected radioactivity per mg. of body weight.

† The values shown in these columns are taken from a previous publication (5).

the tracer than with the large doses (Fig. 2). The decrease in rate of excretion with the dose could be related to a concomitant increase in the radioactivity of plasma and organs (Table V). This in turn was accompanied by an increased utilization of thyroxine with the tracer doses, since it could be calculated from Fig. 1 and Table III that 75 per cent of such a dose became butanol-insoluble within 24 hours, in contrast to only 54 per cent of the large dose (800 γ) previously used (5). Similarly, more butanol-insoluble radioactivity was present in the feces with small than with large doses (Fig. 2). From the excretion of tracer doses, it could be estimated that, of the thyroxine present in the body of the rat at any one time, less than a fifth is likely to be lost in the feces as thyroxine itself.

Finally, the rapid destruction and excretion of thyroxine contrasted with its well known ability to produce a sustained rise of oxygen consumption. The metabolic stimulation must, therefore, be the end result of a series of chain reactions initiated while thyroxine was present in the body.

SUMMARY

1. Female albino rats, weighing 80 to 120 gm. and fed on Purina fox chow, were injected intravenously with doses of I^{131} -labeled thyroxine varying from 0.001 to 20 γ . The 20 γ dose was prepared from DL-diiodothyronine and was labeled in the 3',5' positions; the others were obtained from the plasma of radioiodide-treated rats and consisted of L-thyroxine randomly labeled in any of the four iodine positions. The purity of the radiothyroxine, estimated by isotope dilution, averaged 92 per cent.

The organs and excreta were analyzed for total radioactivity 2, 24, and 72 hours after injection (Fig. 1, Tables I and II), and, in some cases, were fractionated by means of butanol into thyroxine-like and non-thyroxine fractions (Table III).

2. Thyroxine enters all of the organs investigated without being stored in any one. The highest concentrations are found in plasma, liver, and kidney, but a large proportion of the body thyroxine is present in muscle. With time, the radioactivity decreases rapidly in all organs, although at a somewhat faster rate in the plasma than in liver, kidney, skin, brain, spleen, muscle, fat, and perhaps adrenals and pancreas. This difference could be accounted for by a transformation of thyroxine into butanol-insoluble metabolites in these organs (Table III). As an index of the over-all rate of metabolism, the turn-over time of thyroxine was calculated from Table I and found to be about 25 to 35 hours in the rat.

3. While thyroxine, administered in large doses, is to a great extent excreted in the feces as thyroxine-like material, tracer doses are excreted in both urine and feces, mostly in a non-thyroxine form (Fig. 2). Thus, it may be calculated from Fig. 1 and Table III that less than 20 per cent of

the body thyroxine is excreted as such, the rest being converted to its metabolites.

4. The distribution of thyroxine was compared to that of the endogenous thyroid hormone in experiments with tracer radioactive iodide. At 2 hours after iodide injection, the radioactivity is found mainly in the stomach and urine in a butanol-insoluble form (Table IV). After 24 to 72 hours, a sufficient interval for the thyroid to take up radioiodine and release it as thyroid hormone, the organ radioactivity, which is then largely butanol-soluble (Table V), has a distribution similar to that of radiothyroxine. Isotope dilution of the butanol fractions demonstrates the presence of some thyroxine, uncombined in peptide linkage.

From these facts, it is concluded that the results obtained with the smallest doses of labeled thyroxine indicate the pathways of metabolism of the endogenous thyroid hormone.

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PURIFICATION OF HYALURONIDASE

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Hyaluronidase is the generic term applied to a class of enzymes which will hydrolyze hyaluronic acid. The distribution, chemical properties, and biological rôle of these substances have been widely reviewed (1, 2).

Electrophoretic studies¹ were carried out in this laboratory upon hyaluronidase extracted from mammalian testes and purified by various salting-out procedures (3-5). The hyaluronidase activity of each preparation was universally associated with one or more components isoelectric at approximately pH 5.9. Inactive components were isoelectric at more acid levels. This distribution of components with distinct electrophoretic characteristics suggested that their separation might be accomplished by an ethanol fractionation scheme such as that carried out by Cohn *et al.* upon plasma proteins (6). Accordingly, a study was made to determine the effects of variations of pH, alcohol concentration, and ionic strength on the solubility of hyaluronidase. The results of these experiments and their influence on purification procedures are presented in this paper.

Materials and Methods

Determination of Hyaluronidase Activity—The activities of the various enzyme preparations in this study were determined by a modified Kass and Seastone procedure (7). Turbidity reduction of a hyaluronate substrate was measured under standard conditions following a 30 minute incubation period with the enzyme in 0.05 M acetate buffer at pH 6.2 containing additional 0.05 M NaCl.² The activity of an unknown sample was interpolated from a curve of the turbidity reduction values for varying amounts of a standard crude enzyme sample, prepared according to the method of Madinaveitia (3). Dilutions of the standard and unknowns were run simultaneously. The standard enzyme preparation was arbitrarily assigned a turbidity-reducing activity of 1 unit per mg. of protein. As a precautionary measure against possible loss of activity, the standard and unknown enzymes were dissolved at a concentration of 1 mg. per ml. in cold water and subsequently diluted to desired concentrations with cold buffer prior to assay.

Preparation of Starting Material—Crude hyaluronidase, to serve as start-

¹ Tint, H., and Bogash, R., unpublished data.

² Alburn, H. E., and Whitley, R. W., unpublished work.

ing material for the subsequent solubility trials, was prepared on the basis of salting-out procedures described by Madinaveitia (3) and Hahn (4). Decapsulated, ground bovine testes were thoroughly extracted in the cold with equal volumes of 0.1 N acetic acid and the insoluble residue removed. Material which then precipitated between 30 and 69 per cent of saturation with $(\text{NH}_4)_2\text{SO}_4$ at 4° was retained. An aqueous solution of this fraction was dialyzed against cold water until free of sulfate ion. The solution was freed of flocculated material and then dried under a vacuum from the frozen state. The final preparation was approximately twice as active as the arbitrary standard.

Ethanol Treatment—In the following series of experiments several levels of constant ionic strength, with variable pH and alcohol concentrations, were tested. In each run, a 1 per cent solution of the crude hyaluronidase powder was prepared in 500 ml. of cold buffer at a fixed pH (determined by glass electrode at room temperature) and ionic strength, and then absolute alcohol was added gradually, with cooling, until precipitation began. All additions were made by a fine jet to precooled, stirred solutions. The temperatures of the solutions were varied from 0° to -9°, depending upon alcohol content, to reduce the denaturing effects of the solvent. At low alcohol concentrations, the solutions were held within 1° of their freezing point, and for higher concentrations, a maximal temperature of -9° was maintained. At an arbitrarily selected alcohol concentration (expressed as volumes per cent), the solution was permitted to equilibrate with stirring at constant temperature, after which the precipitate was removed by centrifugation under refrigeration. The supernatant was then treated with additional alcohol under similar conditions, and the process was repeated until several successive precipitates had been obtained. Each precipitate was suspended in cold water, immediately shell-frozen, and dried by lyophilization. After accumulation of the dried precipitates from several runs, the individual dry weights of the recovered fractions were determined and each was assayed for enzymatic activity by the standard procedure. Samples of the final supernatant fluids remaining after each run were taken for dilution and freeze-drying to determine residual proteins and activity.

The experiments were performed at each pH unit from 4 to 9 inclusive with buffers as follows: acetate, pH 4 and 5; phosphate, pH 6 and 7; and veronal, pH 8 and 9. The entire series was repeated at each level of ionic strength. In order to maintain constant pH at minimal ionic strength, the solutions of the crude preparation were made in distilled water and the pH adjusted with 0.1 N NaOH or 0.1 N HCl as necessary after each addition of alcohol and separation of precipitate. At higher ionic strengths, further pH adjustments during the runs were usually not required.

In each pH series controls were maintained in order to check any possible coprecipitation of buffer salts at the reduced temperatures in the presence of alcohol. When this phenomenon was observed, the corresponding hyaluronidase samples were dialyzed before lyophilization.

Results

Preliminary Alcohol Fractionations—Figs. 1 and 2 summarize the results of experiments relating variations in the multiphase system to purification and recovery of hyaluronidase. The fractional recovery in each

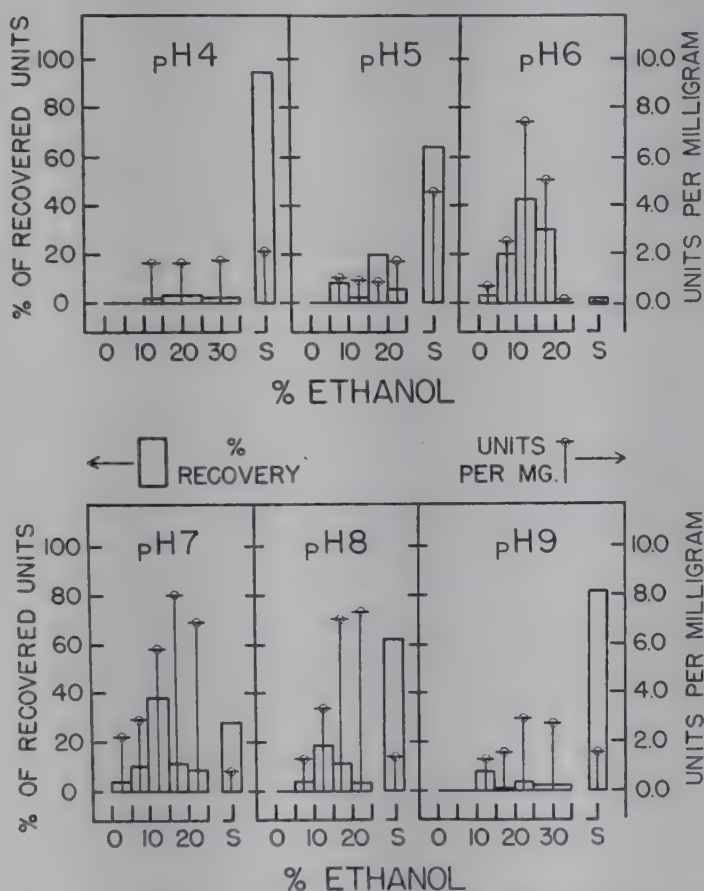


FIG. 1. Relative recovery and purity of ethanol-precipitated hyaluronidase fractions at low temperatures, minimal ionic strength, and constant pH. The supernatant activity is represented by S. The percentage recovery ($\square$) is based on total recovered activity in each pH series; activity ($\circ$), units per mg. of solids.

ethanol precipitate in any given series at constant pH and ionic strength is calculated on the basis of the total recovered activity in that series. Absolute recoveries in the corresponding series, based upon the starting activity, are given in Table I.

Absolute recovery levels were relatively high in all the experiments with the possible exception of that for the most alkaline run. The total activ-

ity losses in the other runs, ranging from approximately 0 to 20 per cent, probably reflect manipulative losses resulting from the handling of small

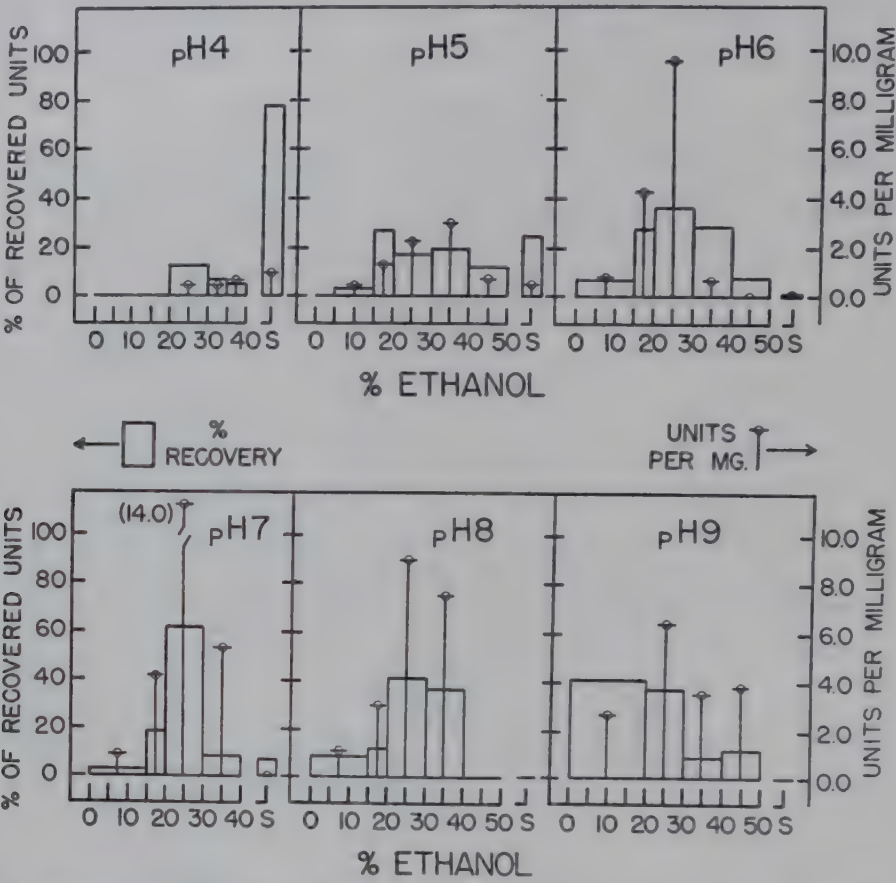


FIG. 2. Relative recovery and purity of ethanol-precipitated hyaluronidase fractions at low temperatures, 0.15 ionic strength, and constant pH. The percentage recovery (□) is based on total recovered activity in each pH series; activity (○), units per mg. of solids.

TABLE I

Absolute Recovery of Initial Enzyme Activity in Ethanol Fractionations at Constant pH and Ionic Strength

Ionic strength	Per cent yield based on starting activity					
	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
Minimal 0.15	97.9	80.0	78.0	85.7	94.5	73.9
	97.7	86.2	99.7	71.9	75.1	70.8

volumes and light precipitates in the various phases of the procedure rather than any adverse effects on activity by the physical environment. The components in the crude hyaluronidase preparation were selectively precipitated under specific conditions of pH, ionic strength, and alcohol

concentration. Optimal recovery and purity of hyaluronidase were obtained in the pH range of from 6 to 8 at minimal (Fig. 1) and 0.15 (Fig. 2) ionic strength. In the latter series, the precipitation of hyaluronidase was somewhat more selective.

Alcohol concentrations required for satisfactory precipitation of hyaluronidase were generally higher at 0.15 ionic strength than at the minimal level. In other experiments at 0.30 ionic strength (0.15 M buffer plus 0.15 M NaCl), hyaluronidase was incompletely precipitated in 50 per cent alcohol solutions. At this concentration, there occurred considerable coprecipitation of buffer salts and losses of over-all activity.

Activity Concentration by Alcohol Fractionation—The trends established in the solubility studies suggested one approach to the purification of hyaluronidase preparations by preliminary precipitation of the impurities at an acid pH (less than pH 6). A solution of 0.578 gm. of hyaluronidase preparation (6.2 units per mg.) was adjusted to approximately 1 per cent solution, pH 5.19, and minimal ionic strength. The addition of alcohol to 15 volumes per cent then precipitated approximately 20 per cent of the original weight of the preparation with negligible hyaluronidase activity. However, further addition of alcohol to 25 per cent precipitated 0.263 gm. of material assaying 5.3 units per mg., representing approximately 40 per cent of the starting activity. This fact suggested that further removal of precipitates at this pH level would yield no further activity enhancement. By altering the pH to 8 and the alcohol strength to 40 per cent, additional light precipitates were obtained with the relatively high activity of 16 units per mg. This purified cut contained, however, only 10 per cent of the starting activity. The final supernatant contained 21 per cent of the original units. Since the bulk of the recovered activity had approximately the same potency as the starting material, preliminary precipitation of the impurities at an acid pH appeared impractical. This run demonstrated that the precipitate of inactive constituents, comprising more than 90 per cent of the starting weight, occluded substantial amounts of the active principle in solution.

Quantitatively greater recovery of concentrated activity was achieved by performing the first precipitation at a more alkaline level. The method and results of this procedure on a comparatively larger scale are shown in Diagram 1.

In order to determine whether additional purification was possible under the particular conditions illustrated in Diagram 1, the precipitated paste of the active fraction was redissolved in cold buffer and subfractions were obtained at several alcohol concentrations. The data for this series (Table II) indicate slight enhancement of activity in the cut between 30 and 40 per cent alcohol; however, this fraction represented approximately one-

third of the activity of the reprecipitated lots. The weighted average of all the reprecipitated fraction was approximately 12 units per mg., indi-

25 gm. at 2 units per mg.
crude enzyme preparation
dissolved in cold phosphate
buffer to give 1.0 per cent
protein solution; pH 7;
 $\mu = 0.15$; total activity =
50,000 units (100 per cent)

Absolute EtOH added
with cooling (0° to
 -6°) to 20 vol. per cent

Ppt. A

3.565 gm. at 3.0 units per mg.;
total activity = 10,700 units
(21 per cent yield)

Supernatant

To 40 per cent EtOH at -9°

Supernatant

33.30 gm. (including salts) at
0.13 unit per mg.; total ac-
tivity = 4340 units (9 per
cent yield)

Ppt. B

2.35 gm. at 13 units per
mg.; total activity =
30,600 units (61 per cent
yield)

DIAGRAM 1. Fractionation diagram for concentration of hyaluronidase activity at neutral pH and 0.15 ionic strength.

TABLE II

Subfractionation of Ethanol Precipitate B (Diagram 1)*

Alcohol concentration	Activity	Total activity	Yield†
<i>vol. per cent</i>	<i>units per mg.</i>	<i>units</i>	<i>per cent</i>
20	13	12,100	40
30	19	10,240	34
40	5	810	3
50	0.8	330	1
Supernatant	0.3	1,235	4

* Total activity in solution, 30,600 units; phosphate buffer pH 7.0, ionic strength 0.15, temperature -6° to -9° .

† Yield in each fraction based on total activity in Precipitate B.

indicating therefore that only limited further purification could be expected by treatment under the same conditions.

Solubility of Ethanol Precipitates in $(\text{NH}_4)_2\text{SO}_4$ Solutions—The product

of the single stage ethanol procedure described in Diagram 1 was also fractionated in concentrated salt solutions. In one experiment, a solution assaying 14 units per mg. of dissolved solids was treated with successive aqueous concentrations of $(\text{NH}_4)_2\text{SO}_4$ at 4° , without pH adjustment. The following absolute yields and purities on a salt-free basis were obtained: at 25.5 per cent salt, 5.4 per cent yield and 2.3 units per mg.; at 33 per cent salt, 16.4 per cent yield and 7.6 units per mg.; and at approximately 71 per cent salt (saturated), 61.8 per cent yield and 41 units per mg. Less than 20 per cent of the total activity could not be accounted for in this run. In another fractionation with different successive salt concentrations, the following fractions were obtained: at 30 per cent salt, 12.5 per cent yield and 3.4 units per mg.; at 40 per cent salt, 17.4 per cent yield and 24 units per mg.; at 50 per cent salt, 33.5 per cent yield and 37 units per mg.; and at 71 per cent salt, 7.7 per cent yield and 9.7 units per mg. The activity not recovered was approximately 30 per cent.

DISCUSSION

In the hyaluronidase preparations studied, the more alkaline components, in this instance associated with the active principle, were least soluble in a neutral to alkaline system, whereas more acid components, associated with enzymatically inactive fractions, were more easily precipitated in acid medium. In these respects the general solubility behavior of the system was analogous to that observed with animal plasmas and sera, and the techniques for the recovery and purification of hyaluronidase activity were similar to those for the γ -globulins (8) and for the antitoxic fractions associated with these globulins (9, 10). Based upon this analogous behavior, experiments are currently in progress to test the possibility of separating additional impurities by reworking the concentrated activity at acid pH levels.

The subsequent refinement of the single stage ethanol precipitate by one salting-out operation with $(\text{NH}_4)_2\text{SO}_4$, bringing 50 to 60 per cent of the recovered solids to a purity level of approximately 40 units per mg., represented an over-all recovery of starting material of better than 30 per cent with a 20-fold increase in activity. Freeman *et al.* (11) have secured 16- to 18-fold purification of hyaluronidase, assaying initially 1000 units per mg. of N,³ in approximately 10 per cent yield, by four successive fractionation stages, including two with ethanol. The alcohol procedures were

³ The fraction of 1000 units per mg. of N, employed by these authors as starting material for subsequent purification, was identical with the starting, crude fraction employed in the present studies. This fraction had a turbidity-reducing activity twice that of the arbitrary standard. On this basis, the numerical conversion factor between the two sets of units is approximately 500.

performed at pH 5 and presumably high ionic strength (starting solutions made of undialyzed $(\text{NH}_4)_2\text{SO}_4$ precipitates in 0.015 M citrate-phosphate buffer containing 0.9 per cent NaCl), necessitating alcohol concentrations of 40 to 55 volumes per cent for precipitation of the activity. In the present experiments, salting-in effects were pronounced at a lower ionic strength (0.30), requiring similarly high alcohol concentrations for hyaluronidase precipitation. Under these conditions, buffer salts were coprecipitated and enzyme denaturation was evident. On the other hand, the optimal conditions established in these solubility studies permit the use of more concentrated protein solutions and less alcohol to secure enhancement of activity in fewer operations and in moderate yield. It should be noted, however, that the purified hyaluronidase fractions obtained in this study were relatively unhomogeneous by electrophoretic analyses.

The writers are indebted to R. W. Whitley and Miss Louise Hall for assays of the enzyme preparations and to J. J. Schaub for technical assistance in the course of these investigations.

SUMMARY

The solubility of hyaluronidase in crude testicular extracts was investigated under various conditions of alcohol concentration, pH, and ionic strength. The precautions usually required to minimize denaturation of active protein principles in this physical environment were observed.

Precipitation of purified hyaluronidase from this system within practical ethanol concentrations was limited to solutions whose ionic strength was less than 0.30.

Hyaluronidase demonstrated maximal insolubility in this system between pH 6 and 8. Non-active components, on the other hand, were generally least soluble at more acid levels. Under optimal conditions, a single precipitation between 20 to 40 per cent alcohol concentration by volume, at pH 7 and 0.15 ionic strength, yielded approximately a 7-fold increase in activity per unit weight. This precipitate contained 61 per cent of the starting activity. Selection of suitable $(\text{NH}_4)_2\text{SO}_4$ concentrations permitted an additional 3-fold enhancement of the activity at better than 60 per cent yield of the intermediate activity. The possibility of a similar increase in purity, by further direct alcohol precipitation as indicated by the data, is discussed.

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SOLUBILITY AND SEDIMENTATION STUDIES ON CERTAIN PENTOSE NUCLEIC ACIDS

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Previous publications from this laboratory (1-3) have described the composition and some chemical properties of certain specimens of ribonucleic acids and of fractions derived from them. Because of the vagueness presently inherent in the term "nucleic acid," it is believed that any specimen which is studied for any purpose should be characterized by many properties in order to facilitate comparison of results obtained in different laboratories. Consequently certain specimens described in other publications were studied by ultracentrifugation and by measurements of solubilities.

Although the examination for heterogeneity by means of solubility diagrams is a well known procedure, apparently only one application has been made to ribonucleic acid (4). Since the present results do not agree with those of the previous paper on two general points, the results will be discussed in detail.

Solubility Studies

Sodium nucleate (2) is dissolved in water or in neutral aqueous salt solution. Different amounts are pipetted into a series of tubes, the volumes are adjusted to a common value with the same aqueous solvent, and a fixed volume of a precipitation agent is added to each tube. The reagents are cooled before mixing. The tubes are closed with rubber stoppers and are placed in a rocking apparatus in a cold room. At intervals the precipitates are removed and the contents of total phosphate in the solutions are determined by the method of Fiske and Subbarow (5). The precipitating agent was ethyl alcohol or acetone, with or without acetic acid. The aqueous sodium nucleate solutions were at pH 6.

Although air interfaces can cause denaturation of proteins during solubility measurements, they apparently did not interfere in these studies, since solubilities were observed to be constant over periods as long as 3 weeks.

Difficulties were encountered in the separation of some precipitates, as illustrated by the system of Diagram A, Fig. 1. The more concentrated supernatants, after 15 minutes of centrifugation at $16,000 \times g$, were tur-

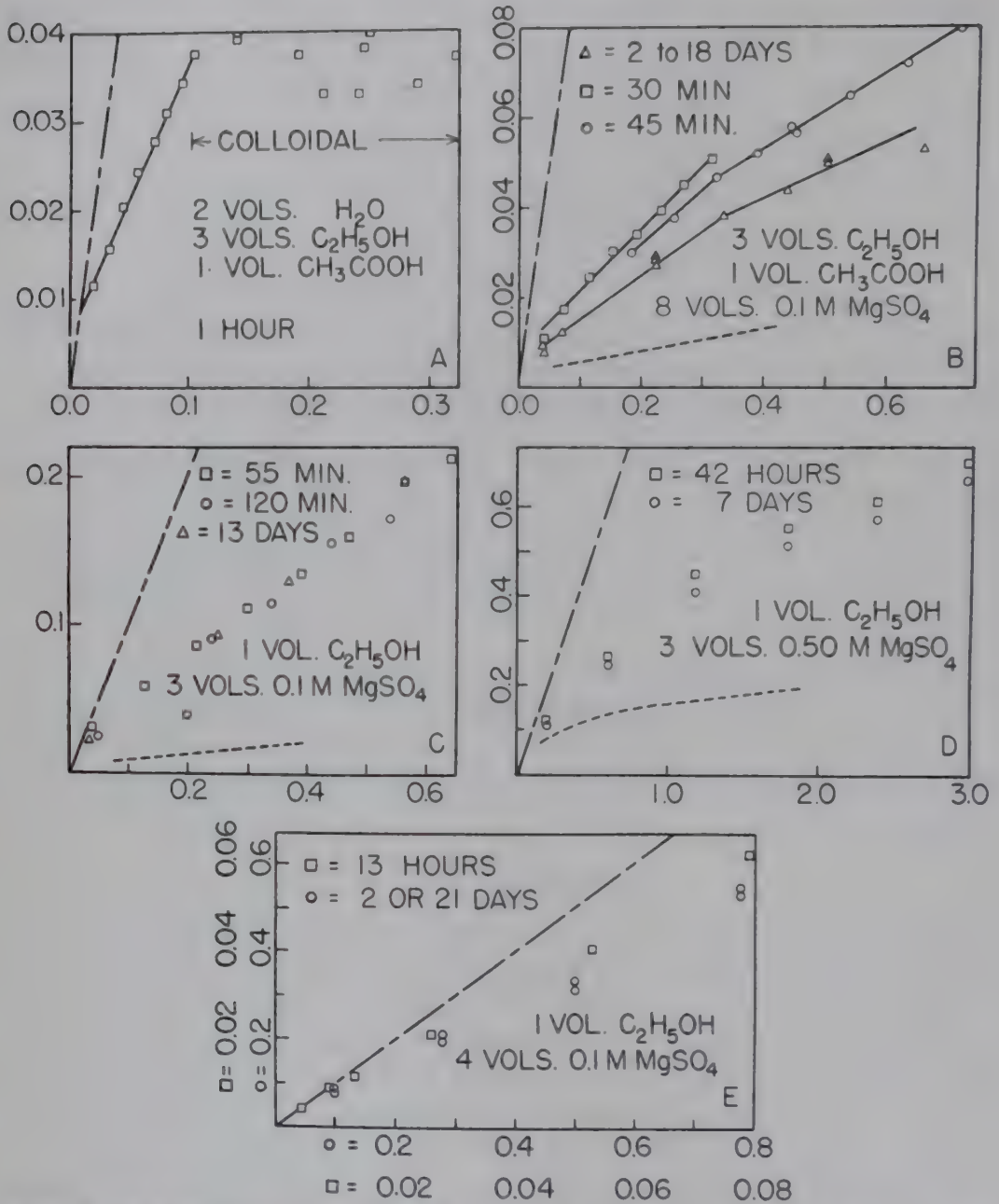


FIG. 1. Solubility curves of purified, dialyzed ribonucleic acid from yeast. Equilibration of precipitates with solvents for the given times at 5° and with constant shaking. All the abscissas are mg. of total P per ml. All the ordinates are mg. of soluble P per ml. The long and short dash lines represent 100 per cent solubility; the short dash lines represent solubility of precipitates after removal of original saturated solvent and replacement by an equal volume of fresh solvent.

bid; after 45 minutes they were clear, but analyses of samples removed from the top and from the bottom of each supernatant revealed strong gradients of phosphate concentration. The points in the colloidal range of Diagram A were obtained by 75 minutes of centrifugation at 5°.

Magnesium sulfate was tested for its influence on the coagulation of the colloid. From the results it is believed that the use of the salt, in excess or at least at saturation, will prove a valuable reagent for the preparation and fractionation of nucleic acids.

According to Chantrenne (4), the solubilities of barium, lanthanum, lead, and magnesium nucleates were not reproducible. The explanation may lie in the fact that different amounts of nucleate in each tube of a series would precipitate different amounts of cations, and the resulting solvents would have different ionic strengths. In the present studies, if it is assumed that 1 magnesium ion precipitates with two phosphate groups, then in no case was more than 12 per cent of the total magnesium precipitated, and the ionic strengths of the resulting supernatants of a series were essentially constant. The sodium nucleate, in the presence of magnesium sulfate, was more than 10 per cent soluble in water at 20° and at neutrality. A flocculent precipitate formed on the addition of a small amount of alcohol to a dilute solution. The precipitate could be repeatedly dissolved in water and reprecipitated with alcohol in the presence of magnesium ions. Thus, the classical procedure of purification by precipitating in acid and dissolving in alkali can be avoided.

The diagrams of Fig. 1 are representative of the data obtained with one specimen of ribonucleic acid from yeast. In general the magnesium precipitates were separated by centrifugation for 30 minutes at 5° and at $2000 \times g$. Recentrifugation of some of the resulting supernatants at $16,000 \times g$, followed by filtration, removed no more phosphate. The establishment of equilibrium was indicated by the constancy of solubility with time and by the approach to equilibrium both from above and for a few points from below saturation. The present results are in disagreement with those of Chantrenne (4) with respect to the length of time required to reach equilibrium. Chantrenne allowed only 15 minutes for equilibration, whereas Diagram B (Fig. 1) indicates that many hours are required for the equilibration of precipitates formed in acid. However, Diagram C indicates that no more than 1 hour is required for the equilibration of water-soluble magnesium salts. The same contrast was noted in other experiments. The difference between the two curves of Diagram D corresponds to about twice the standard deviation of the standard curve for the phosphate determination; the difference is not necessarily significant. In one experiment in which magnesium nucleate was precipitated with alcohol, 1 portion of the original aqueous solution was adjusted to pH 5, while another was adjusted to pH 8. The two corresponding precipitates had the same solubility.

An interesting feature of the precipitation is illustrated by Diagrams C and E (Fig. 1). The corresponding solvents contain 25 and 20 volumes

per cent of alcohol; two-thirds and one-quarter of the phosphate are precipitated, respectively, independently of the concentration. Even under such mild conditions, the precipitates are flocculent and separate readily. The nucleate is completely soluble to a concentration of at least 0.7 mg. of P per ml. if the solvent is 15 volumes per cent of alcohol in 0.1 M magnesium sulfate.

Without enumerating the specific points of evidence, it is clear from Fig. 1 that the specimen of nucleic acid is a mixture of at least three, and probably more, components. Diagram B shows the only discontinuity of slope which has been observed with that specimen. In agreement with Chantrenne (4), the solubilities of the precipitates are all very low, regardless of the proportion of phosphate in the precipitate and regardless of the solvent. The intercepts on the axes of the ordinate range from 1 to 30 mg. of P per liter.

Surprisingly, the thoroughly dialyzed residue prepared from the nucleic acid by the action of ribonuclease (3) contains a "component" which forms a magnesium salt that is only sparingly soluble in water at neutrality. This magnesium salt contains one-third of the phosphate of the residue, and its solubility is 80 mg. of P per liter. Solubility diagrams obtained by the precipitation of sodium salts of the residue with alcohol gave further evidence of heterogeneity.

Sedimentation-Velocity Measurements

The observations were made in a Spinco ultracentrifuge. Eight measurements were made of the apparent specific volume of the sodium salts at concentrations from 0.2 to 2 per cent. The value obtained was 0.48 ± 0.02 and was independent of the concentration. The materials contained from 1 to 2 per cent water. Viscosity measurements were made in an Ostwald type of viscosimeter with an average shear gradient of about 300 sec^{-1} . Such measurements were translated into frictional ratios by the charts prepared by Oncley (6), with the assumption of 20 per cent hydration and rod-shaped particles.

One run in the ultracentrifuge was made on each of three materials at concentrations of approximately 1 per cent in acetate buffer, pH 4.6, and ionic strength 0.15. The materials were a nucleic acid from yeast (2), a residue from that nucleic acid (3), and a residue from a pancreatic nucleic acid (3). The boundaries sedimented slowly and spread rapidly; in view of the proved heterogeneity, it was considered futile to attempt more precise physicochemical characterization of the materials. However, semi-quantitative results of interest were obtained from the three runs.

The schlieren diagrams for the nucleic acid from yeast are bell-shaped

and somewhat skewed to the centripetal end. The sedimentation constant corresponding to the movement of the peak of the curve is 2.1s. The viscosity increment (7) is 7.8. The molecular weight is approximately 6500.

A zone of radially constant concentration remained between the bottom zone and the boundary zone up to 102 minutes after the attainment of full speed. These diagrams, at 38, 70, and 102 minutes, were enlarged and the second and fourth moments were computed for the areas centrifugal to the peaks. Fourteen to eighteen intervals were used. After correction to the actual cell dimensions, diffusion constants were computed from the second moments by the formula $D = \sigma^2/2t$. The values of D at 38, 70, and 102 minutes are 2.2, 2.0, and 2.2×10^{-6} . The value of D computed from the sedimentation constant and from the viscosity is 1.6×10^{-6} . The ratio, $(2.1/1.6)^{\frac{1}{2}}$, is 1.14, which is a relative error within the resolution of the boundary curves. Furthermore, each fourth moment was divided by the square of the corresponding second moment. The resulting quotients are 2.9, 2.9, and 2.7. The value expected for a normal curve is 3.0. These data are presented as evidence for believing that the bulk of the material is rather homogeneous with respect to molecular weight and that the spreading of the boundary during the sedimentation was largely due to diffusion. The trailing edges of the boundary curves undoubtedly indicate the presence of a minor proportion of smaller fragments.

The schlieren diagrams of the residue prepared from the nucleic acid from yeast, although initially showing a distinct peak, developed very broad, flat boundary zones. However, there are two features of interest. The first is that the advancing edge of the boundary zone of the residue has the same shape and moved at the same rate as the advancing edge of the boundary zone for the original nucleic acid. Thus, it appears that some of the original molecules were undegraded with respect to molecular weight following the exhaustive action of ribonuclease. The second feature is that the rate of movement of the trailing edge of the boundary zone indicates that the bulk of the material has a molecular weight greater than approximately 2000, which thereby gives better definition to the expression "non-dialyzable" as it is used in this series of papers.

The schlieren diagrams of the residue from the pancreatic nucleic acid resemble the diagrams of the original nucleic acid from yeast. The sedimentation constant corresponding to the movement of the peak is 2.8s. The viscosity increment is 6.1, and assuming 20 per cent hydration, the molecular weight is approximately 10,000.

The second moments of the areas of the curves centrifugal to the peaks were used as before to infer that the bulk of the material is homogeneous with respect to molecular weight, although there is a minor proportion of smaller fragments.

In spite of the nearly equal compositions of the residues from pancreas and from yeast, the molecular weight distributions are quite different.

The authors wish to express their thanks and appreciation to Dr. H. K. Schachman and to Mr. W. F. Harrington for the ultracentrifugal and viscosimetric determinations.

SUMMARY

Certain ribonucleic acids, which had been the object of previous studies, were examined for heterogeneity by means of solubility diagrams. Molecular weights were estimated by measurements of sedimentation velocities and of viscosities.

A specimen of ribonucleic acid from yeast, although heterogeneous, was largely composed of molecules whose weights were approximately 6500.

After subjection of the ribonucleic acid from yeast to the prolonged action of ribonuclease during dialysis, the recovered residue was found to be heterogeneous, with a dispersion of molecular weights approximately in the range from 2000 to 6500.

A residue prepared from the ribonucleic acid from pancreas was largely composed of molecules with weights of approximately 10,000.

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A SPECTROPHOTOMETRIC METHOD FOR THE MICRO-DETERMINATION OF HEXOSAMINES

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The only method hitherto available for the determination of hexosamines, which is based on a specific color reaction, is that of Elson and Morgan (1) which requires more than 20 γ of material. A new spectrophotometric procedure has been elaborated for the determination of hexosamines in polysaccharides, based on a principle completely different from that of the Elson and Morgan method and having the advantage of being considerably more sensitive. As little as 5 γ of hexosamine can be determined.

EXPERIMENTAL

Principle of Method

Hexosamines, when deaminated by nitrous acid, are converted to 2,5-hexose anhydrides with simultaneous Walden inversion at carbon 2. The anhydrohexoses yield characteristic colors with indole in dilute hydrochloric acid. The method depends upon the difference in intensity of color formed when the indole reaction is applied to a hexosamine solution before and after deamination.

Procedure

Deamination of Hexosamine—Thoroughly cleaned test-tubes of equal diameter and wall thickness are used. To 0.5 cc. of the unknown, 0.5 cc. of a 5 per cent solution of sodium nitrite, c.p., and 0.5 cc. of a 33 per cent solution of acetic acid, c.p., are added. The tubes are then shaken and left standing for 10 minutes, at which time the deamination is completed. The excess nitrous acid is then removed by adding 0.5 cc. of a 12.5 per cent solution of ammonium sulfamate, c.p., and repeatedly shaking the mixture for periods of 30 minutes.

Indole Reaction—To 2 cc. of a solution which contains 5 to 100 γ per cc. of the deaminated hexosamine, 2 cc. of 5 per cent hydrochloric acid and 0.2 cc. of a 1 per cent solution of indole in alcohol are added. The tubes are then immersed for 5 minutes in a vigorously boiling water bath. An intensive orange color and a slight turbidity appear. To remove the latter, 2 cc. of alcohol are added and the tubes are shaken.

Absorption Spectrum of Reaction Product

At least two different products are formed in the indole reaction. When the reaction mixture is shaken with chloroform, a pink color is taken up while a brown color remains in the water phase. The separation is of no advantage because absorption curves of the two components show insufficient difference in the position of their peaks. The absorption curve of the mixture shows a maximum at $492\text{ m}\mu$ with the Beckman spectrophotometer. The absorption decreases sharply on both sides of the maximum and levels off at 400 and $520\text{ m}\mu$ respectively (see Fig. 1).

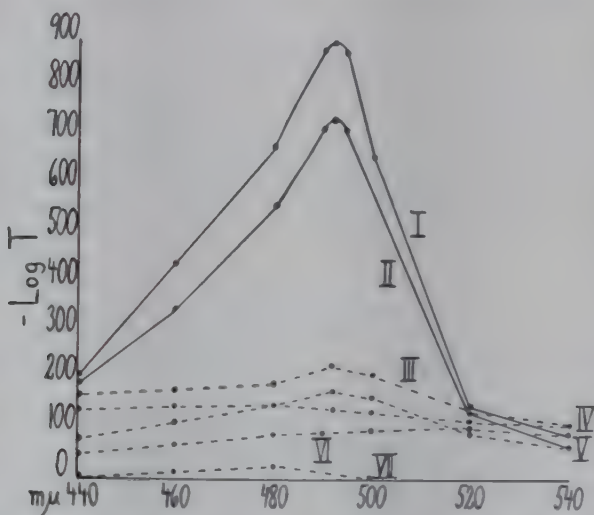


FIG. 1. Absorption spectra of compounds derived from hexosamines by deamination and of other sugars in the indole reaction. Compound I, glucosamine 8 mg. per cent; Compound II, chondrosamine 7 mg. per cent; Compound III, galacturonic acid 50 mg. per cent; Compound IV, mannose 50 mg. per cent; Compound V, ribose 100 mg. per cent; Compound VI, fructose 50 mg. per cent; Compound VII, xylose 50 mg. per cent.

Specificity of Reaction

Hexosamines yield no color without prior deamination. Nitrogen-free carbohydrates react with indole and hydrochloric acid and some of them yield a maximum of absorption at $492\text{ m}\mu$, but the absorption at this wavelength is much lower than in the case of deaminated hexosamines. The indole reaction of true carbohydrates, however, is not affected by treatment with nitrous acid. On the other hand, serum albumin at 0.2 per cent and ascorbic acid at 0.01 per cent, when treated with nitrous acid, show an increase of absorption at $492\text{ m}\mu$ in the indole reaction. However, the resulting absorption curves show no peak at $492\text{ m}\mu$. To avoid any interference of these substances in quantitative determinations of hexosamine, we determine the difference of density observed at 520 and

492 $m\mu$ respectively, the increase of this difference after the deamination procedure being a measure of the amount of hexosamine. Serum albumin and ascorbic acid do not show such an increase.

Quantitative Determination of Hexosamines

As can be seen in Table I, $D_{492} - D_{520}$ for deaminated hexosamines is proportional to the amount of hexosamine in the concentration range of 10 to 100 γ per cc. of the solution. The values of $D_{492} - D_{520}$ for glucosamine and for chondrosamine do not differ significantly. It can be seen from Experiment VIII that serum albumin, hydrolyzed for 1 hour with 2 N sulfuric acid, did not affect $D_{492} - D_{520}$ of glucosamine. The density increment of deaminated glucosamine was not changed by the presence of 0.1 per cent of glucose or 0.02 per cent of glucuronic acid. It is therefore possible to determine hexosamine quantitatively in the following way. To determine the increase of $D_{492} - D_{520}$, two 0.5 cc. samples of the unknown are deaminated as described above. To each of two other samples, 1.5 cc. of a mixture of equal volumes of solutions of 5 per cent sodium nitrite, 33 per cent acetic acid, and 12.5 per cent ammonium sulfamate are then added; these serve as controls without deamination. Two pairs of samples of a standard solution of hexosamine of appropriate concentration are run in the same manner. The indole reaction is carried out on all eight mixtures and on a water blank, as described above. $D_{492} - D_{520}$ for the non-deaminated solutions is subtracted from the corresponding values for the deaminated unknown and standard solutions. The difference for the unknown, divided by that observed for the standard solution, gives the relative concentration of hexosamines in the two solutions.

Determination of Hexosamines in Polysaccharides

As hexosamines in polysaccharides are present in general in the form of their acetyl derivatives, the determination of hexosamine must be preceded by deacetylation. Apart from this, complete hydrolysis of glycosidic linkages is apparently necessary for this determination. As a rule the splitting of glycosidic linkages requires longer hydrolysis than is necessary for the deacetylation, which is completed after heating for 1 hour with 1 N hydrochloric acid. This was shown in the case of blood group substance A from hog mucosa. Complete splitting of glycosidic linkages in this preparation was achieved by a 2 hour hydrolysis with 2 N hydrochloric acid at 100°. This was indicated by the fact that the reducing power of the hydrolysate reached a maximum at this time. The Elson-Morgan procedure indicated that the hexosamine content of the hydrolyzed compound was 33.6 per cent. Practically the same value was ob-

TABLE I

Densities at 492 and 520 mμ and Their Differences for Various Substances, with Indole Reaction

Experi- ment No.	Substance	Concen- tration	$D_{492} \times$ 1000	$D_{520} \times$ 1000	$D_{492} -$ $D_{520} \times$ 1000	$D_{492} -$ $D_{520} \times$ 1000
			Deaminated		Deami- nated	Not de- aminated
		mg. per cent				
I	Glucosamine	1.25	159	32	127	6
	"	2.5	299	54	245	3
	"	5.0	632	116	516	24
	"	10.0	1225	195	1030	25
II	"	10.0	985	156	829	0
	Chondrosamine	10.0	1046	155	891	13
	Acetylglucosamine (hydro- lyzed 1 hr. with 2 N sulfuric acid)	12.0	940	148	792	18
III	Mannose	50.0	2	-2	4	7
	Glucose	100.0	28	23	5	8
	Ribose	100.0	200	80	120	105
	Glyceraldehyde	50.0	3000	570	2430	2395
IV	Glucosamine	5.0	1515	280	1235	4
	" +	5.0				
	Glucose	100.0	1590	340	1250	12
V	Glucosamine	10.0	2900	640	2260	8
	" +	10.0				
	Glucuronic acid	20.0	2900	630	2270	13
VI	Blood group substance (hy- drolyzed 2 hrs. with 2 N hy- drochloric acid)	5.3*	548	124	424	25
	Glucosamine	2.0	588	127	461	25
	Blood group substance (hy- drolyzed 1 hr. with 2 N sul- furic acid)	5.1*	365	72	293	7
VIII	Glucosamine	1.9	485	114	371	29
	Serum albumin (hydrolyzed 1 hr. with 2 N sulfuric acid)	100.0	24	12	12	10
	Serum albumin as above + Glucosamine	8.3	1055	170	885	24
	Glucosamine	8.3	1070	185	885	4
IX	Serum albumin (hydrolyzed 3 hrs. with 2 N sulfuric acid)	100.0	90	70	20	29
	Ascorbic acid	10.0	82	78	4	-1

* The solutions of blood group substance A in Experiments VI and VII contained 1.73 mg. per cent and 1.66 mg. per cent of hexosamine respectively, according to the determinations by the method of Elson and Morgan.

tained with the indole method as can be calculated from Experiments VI and VII in Table I. When the indole reaction was carried out on a solution of blood group substance A, which had been hydrolyzed for 1 hour only (sufficiently to split off the acetyl group of glucosamine), it yielded a value for the content of hexosamine which was only 89 per cent of that expected.¹ After hydrolysis, it is necessary to neutralize the hydrolysate for deamination.

DISCUSSION

2,5-Anhydrohexoses behave in a similar manner to that of the true sugars in most of the color reactions of sugars based on the breakdown by strong acids. In some of these reactions, as, for example, the cysteine (2) and diphenylamine (3) reaction, there is a striking parallelism between the behavior of the deaminated hexosamines and fructose. In the so called basic cysteine reaction, the primary breakdown product (probably hydroxymethylfurfural) is formed in much higher amounts from fructose and deaminated hexosamines than from aldohexoses. In the diphenylamine reaction the peak of the color development is reached much more rapidly with fructose than with aldohexoses. Deaminated hexosamines behave like fructose. This parallelism could be due to the fact that the formation of furfural and its homologues from hexoses requires a rearrangement from the 1,4(butylene oxide) or 1,5(amylene oxide) ring to the 2,5-butylene oxide ring. The latter is already present in the 2,5-anhydrohexoses and to a certain extent in fructose, which is known to exist in solution to a considerable extent in the fructofuranose form.

In principle, the increase in the reactivity of the hexosamines after deamination in any of the above mentioned reactions could be used for the determination of these compounds. Our own attempts in this direction showed, however, that these reactions are less reliable and useful for this purpose than the indole reaction.

The determination of hexosamines in polysaccharides by the indole reaction, as well as by that of Elson and Morgan (1), requires complete hydrolysis of the polysaccharide. Even if this hydrolysis is carried out under conditions which do not destroy hexosamines in pure solutions, it is not certain whether destruction of the hexosamine molecule occurs when it is combined by glycosidic linkages with other sugars. Under these circumstances it may be of advantage to compare the values for the hexosamine content obtained by the Elson and Morgan method with those obtained by the indole reaction. In the case of acid mucopolysaccharides

¹ We are greatly indebted to Dr. Elvin A. Kabat for the preparation of blood group substance A and to Dr. Karl Meyer for the preparation of hyaluronic acid, chondrosamine hydrochloride, and chondroitinsulfuric acid.

(hyaluronic acid; chondroitinsulfuric acid)¹ it was found that the indole reaction gave too low values after a hydrolysis which was sufficient to yield nearly theoretical values in the Elson and Morgan procedure.² As hexosamine added to the hydrolysate was completely recovered in the indole reaction, this discrepancy could be due to incomplete hydrolysis of the polysaccharides and requires further investigation.

SUMMARY

1. A new characteristic color reaction of hexosamines based on their deamination to 2,5-anhydrohexoses is described.

2. The reaction permits the quantitative determination of as little as 5 γ of hexosamine.

3. The use of the method for the quantitative determination of hexosamine in polysaccharides is discussed.

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² Unpublished data.

THE INFLUENCE OF BILE ON THE ALKALINE PHOSPHATASE ACTIVITY OF INTESTINAL LYMPH*

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A marked increase in the activity of alkaline phosphatase of intestinal lymph following feeding has previously been found in the rat (1). Because this increase was much greater than that found in the plasma of the intact rat after feeding and because the activity of this enzyme decreased to very low levels in the plasma of rats from which lymph was being drained, it appeared that the small intestine is normally an important source of the alkaline phosphatase of the plasma in this animal. It has been suggested that the intestine might also be an important source for the elevated levels found in the plasma of animals with obstructive jaundice (2). Dalgaard (3) has recently shown, however, that this is unlikely, since elevated plasma levels could be produced by ligation of the common bile duct in dogs which had previously undergone enterostomy.

We have reinvestigated the effect of fat-containing and fat-free meals on the alkaline phosphatase activity of intestinal lymph of the rat under somewhat more favorable conditions for both enzyme measurement and lymph flow. The increase appears to be considerably larger after fat-containing meals than after fat-free meals in the normal rat. If, however, bile is diverted from the intestine either by a biliary fistula or by ligation of the bile duct, an increased amount of alkaline phosphatase is not released into the intestinal lymph after feeding.

Methods

Male rats of the Sprague-Dawley strain, weighing approximately 200 gm. and maintained on Friskies, were used in these studies. They were fasted approximately 20 hours prior to operation, when a cannula was inserted into an intestinal lymphatic trunk in the mesentery just prior to its entrance into the cisternal chyli, as previously described (4). The rats were placed in small cages (5) with free access to water to which sodium chloride had been added to make a 0.4 per cent solution. The salt was included in an attempt to compensate in part for the loss of salt in the

* Read at the 116th meeting of the American Chemical Society, Atlantic City, September 18-23, 1949.

lymph, which was collected continuously, and to minimize clotting in the cannula by maintenance of a good flow of lymph during the first 24 hour period of collection, when clotting is most likely to occur (6). On the day after operation the rats were given a meal by stomach tube and lymph was collected in three periods, each of 2 hours, at the end of which time a second meal was given and lymph was collected for an additional 18 hour period. Blood was removed by cardiac puncture for comparison with the lymph at this time.

The rats were fed either fat-free or fat-containing meals. The fat-free meal consisted of 5 gm. of the fat-free diet mixed with 5 ml. of water in the Waring blender (1). The fat-containing meal consisted of the same plus 1 ml. of corn oil. With the administration of two meals a day the weight of the rat did not decrease more than about 10 per cent after the operation. The animals appear to be in good condition, although we have been unable to compensate entirely for the continuous loss of lymph.

Additional operative procedures were carried out in some rats at the time the cannula was inserted into the lymphatic vessel. These involved either ligation of the bile duct or introduction of polyethylene tubing into the duct to permit continuous collection of bile as well as lymph. These rats were fed fat-containing meals, and lymph was collected from them at the same intervals described previously.

For determination of alkaline phosphatase activity 0.2 ml. aliquots of lymph or plasma were incubated with 5 ml. of Bodansky's substrate (7) to which magnesium chloride in 0.02 M concentration had been added. The phosphorus liberated was measured by the method of Fiske and Subbarow (8). 1 unit of phosphatase refers to 1 mg. of liberated phosphorus. The daily secretion of phosphatase during the 24 hour feeding period is the sum of the secretions for the individual periods, which were obtained by multiplying the concentration or activity per ml. by the volume, while that for the fasting period between the operation and the first meal was calculated to a 24 hour period when necessary for comparison.

Results

The average hourly secretion of alkaline phosphatase in the intestinal lymph of eleven normal rats during the fasting period, from the time of operation until the first meal was given on the following morning, was 0.22 unit. This increased at varying rates, probably related to the differences in the emptying time of the stomach, in all the rats receiving the fat-containing meals. Maximal values for activity of the enzyme were usually found from the 4th to the 6th hour after the fat meal and were about 8 times the mean value of the control period.

When rats with ligation of the bile duct or with biliary fistulas from

which all the bile was being drained continuously were given the fat-containing meals, there was little if any change in the concentration or average hourly secretion of alkaline phosphatase in the intestinal lymph.

In normal rats fed the fat-containing meals the volume of lymph collected during the 24 hour feeding period was twice as great as in the fasting period (Table I). The increased lymph flow is undoubtedly related both to the food given and to the volume of water given with it. An increase of similar magnitude was found in another group of normal rats fed fat-free meals. When bile was excluded from the intestine by biliary fistula, no such increase of volume was found. This is not surprising, however, since these rats were losing an additional volume of fluid daily of 22.9 ml. as bile. Smaller volumes of lymph were generally found

TABLE I

24 Hour Secretion of Alkaline Phosphatase in Intestinal Lymph

Plus-minus figures are the standard error of the mean.

No. of rats	Group	Meals	Lymph volume		Alkaline phosphatase	
			Fasting	Fed	Fasting	Fed
			ml.	ml.	units	units
11	Normal	Fat-containing	14.4 $\pm$ 2.3	34.6 $\pm$ 3.1	5.2 $\pm$ 0.4	37.8 $\pm$ 4.0
9	"	Fat-free	15.9 $\pm$ 1.2	32.1 $\pm$ 5.0	4.1 $\pm$ 0.4	9.4 $\pm$ 0.7
7	Bile excluded, fistula	Fat-containing	12.4 $\pm$ 3.2*	15.0 $\pm$ 2.4	2.3 $\pm$ 0.5*	3.0 $\pm$ 0.8
9	Bile excluded, ligation	"	10.5 $\pm$ 2.7	21.7 $\pm$ 4.0	3.0 $\pm$ 0.7	6.0 $\pm$ 0.9

* Fasting values from three rats only.

also during the feeding period in rats with ligation of the bile duct than in normal rats. The mean total secretion of alkaline phosphatase in the 24 hour feeding period in normal rats fed the fat-containing meals was 37.8 units in contrast to 5.2 units during the fasting period. Thus a 7-fold increase in the amount of enzyme was found associated with only a 2-fold increase in the volume of lymph. In normal rats fed fat-free meals the secretion of this enzyme was doubled during the feeding period as compared with the fasting period, as was the volume of lymph. The output of enzyme in the lymph of rats when bile was excluded from the intestines was as low as, or even lower than, that found in the normal rats after feeding of the fat-free meals. In the rats with biliary fistulas the average amount of enzyme secreted in the 24 hour feeding period was only 3.0 ± 0.8 (s.e.), which was about a twelfth of that found in the normal rats. This could not be attributed to loss of the enzyme in the

bile, since the amount of alkaline phosphatase found per day in the bile was approximately 1 unit only.

The concentration of alkaline phosphatase in the last sample of lymph is shown opposite that of the plasma taken at the end of the period (Table II). The high level in the lymph is found only in the normal rats fed a high fat meal. Low values were found in the plasma of the normal rats from which all the intestinal lymph had been drained; seven of the eleven fat-fed rats had values between 4.8 and 12.1, but four had from 26 to 41 units, which suggests an incomplete collection of lymph from these four rats. Samples of plasma taken 2 and 6 hours after rats with ligations of the bile duct had received a fat-containing meal showed higher concen-

TABLE II

Concentration of Alkaline Phosphatase in Last Samples of Lymph and Plasma

No. of rats	Group	Meals	Alkaline phosphatase, units per 100 ml.	
			Intestinal lymph*	Plasma
11	Normal	Fat-containing	124 $\pm$ 13	16.2 $\pm$ 3.9
9	"	Fat-free	30.9 $\pm$ 2.2	5.2 $\pm$ 0.6
7	Bile excluded, fistula	Fat-containing	22.2 $\pm$ 2.9	21.0 $\pm$ 5.2
8	Bile excluded, ligation	"	22.7 $\pm$ 3.2	51.4 $\pm$ 5.9

* The lymph samples were collected for an 18 hour period from 6 to 24 hours after the first meal, except in the group of rats with ligated bile ducts. In the latter group collection was made 2 to 6 hours after the first meal.

trations of enzyme than did the lymph. The concentration in the lymph was as low as in fasted rats, while that in the plasma was very high for rats with external lymph drainage. This was undoubtedly owing to the obstructive jaundice with its attendant liver damage and increased amount of alkaline phosphatase in the liver. Low values were found for the lymph of rats with biliary fistulas and also in the plasma in five of seven of these rats.

Comment

The average daily output of alkaline phosphatase in the normal rats of 37.8 units in the present study is much higher than the average of 8.7 units found in a series of five rats given a high fat meal, which was reported previously (1). The higher value now found is to be attributed partly to the improved substrate used, which contained Mg^{++} as an activator, and sodium β -glycerophosphate only as a substrate, instead of the mixed salt containing sodium α -glycerophosphate which we used

previously. Each of these changes in substrate produces approximately a 30 per cent increase in the determined enzyme activity. Other factors responsible for the present high values are the administration of two meals in the 24 hour period instead of one, and the use of 0.4 per cent sodium chloride in the drinking water, which produces an increased flow of lymph. The increased salt intake seemed desirable for the maintenance of a good lymph flow, particularly in the rats with ligation of the common bile duct or biliary fistula. The magnitude of the increase of alkaline phosphatase secreted in the lymph after fat-containing meals as compared with that found after fat-free meals is much more pronounced in this study, with the more favorable conditions for lymph flow and enzyme measurement, than was found in our earlier experiments. It now appears that the increase in the amount of enzyme secreted after the fat-free meals can be explained as due to a higher volume of lymph of unchanged composition. The importance of fat in the diet in the maintenance of high levels of serum alkaline phosphatase has been demonstrated previously by a number of investigators (9-12).

Very little if anything is known as to the significance of the presence of an enzyme such as alkaline phosphatase in circulating lymph and blood, or the mechanism by which the amount circulating increases in both fluids and especially in lymph during the digestion of a meal. These studies demonstrate that the presence of bile in the intestine is involved in some way in the production of an increased output of alkaline phosphatase from the small intestine into the intestinal lymph during the absorption of foodstuffs. Thus the lowest output of phosphatase was found in the rats with biliary fistulas, in which there was a complete absence of bile in the intestine. There was also a very low output in rats with obstructive jaundice produced by ligation of the common bile duct. Observations of the intestinal lymph of the rats with ligation of the bile duct or biliary fistulas show that the lymph has a low fat content and is unusually clear in the fasting rat. After a fat-containing meal the fat content of the lymph from intestines without bile does increase but much less rapidly and not as much as in normal rats after the feeding of fat. The alkaline phosphatase content of the lymph, however, is not correspondingly increased.

Dalgaard (11) has shown that there is an increase of alkaline phosphatase of the serum of rats when the bile ducts are ligated, but that the increase is smaller than that found in rabbits, dogs, or man, and often no greater than can be produced with feeding of olive oil. The observation of higher values in plasma than in intestinal lymph of rats with ligations of the bile duct indicates that these plasma levels are not dependent on enzymes coming from the intestinal mucosa by way of the lymph. In this respect the rat resembles the dog (3). It is probable that the increased

amount of alkaline phosphatase found in the plasma of rats with ligation of the bile duct comes from the liver, in which the concentration of alkaline phosphatase is greatly increased.

SUMMARY

The increase of the alkaline phosphatase of intestinal lymph following feeding of a fat-containing meal is abolished or greatly diminished in rats with ligation of the bile duct or biliary fistula. Thus it appears that bile in the intestine is somehow involved in the transport or release of alkaline phosphatase from the intestinal mucosa, as well as in the absorption of fat. The phosphatase content of the intestinal lymph of fat-fed rats increases with increased fat absorption, but not when bile is absent from the intestine.

In the rats with obstructive jaundice, higher concentrations of the enzyme are found in plasma than in intestinal lymph, suggesting that the intestinal mucosa is not responsible for this increased amount of enzyme in the plasma.

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THE UPTAKE IN VITRO OF C¹⁴-LABELED GLYCINE, L-LEUCINE, AND L-LYSINE BY DIFFERENT COMPONENTS OF GUINEA PIG LIVER HOMOGENATE*

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We have reported (1) that L-lysine labeled with C¹⁴ can be incorporated into the proteins of guinea pig liver homogenate under two different conditions. In the one case the enzyme used was the whole homogenate, the optimum pH was near 6.2, there was an obligatory requirement of calcium, and the incorporation was independent of oxygen. This set of conditions is designated below as the "acid calcium" condition. In the other case the enzyme system was the precipitate obtained by centrifuging the homogenate diluted 15-fold with Ringer's solution at 2500 $\times g$, the optimum pH was near to 7.3, the reaction was accelerated a little by calcium but the presence of calcium was not obligatory, and the incorporation was a little less under nitrogen than under oxygen. This set of conditions is designated below as the "alkaline" condition.

We have carried the study further. The homogenate was separated into four fractions by differential centrifugation, which are designated below as Sediments I, II, and III, and supernatant, and we investigated the ability of each of these fractions to incorporate lysine into the proteins under the two sets of conditions. It was found that all three sedimented fractions incorporated lysine into the proteins under the "alkaline" condition, and that the incorporation under the "acid calcium" condition was confined to the supernatant fraction.

We have investigated also and report here the incorporation of C¹⁴-labeled glycine and leucine into the proteins by the three sedimented fractions, the absence of interaction of amino acids on their individual uptake, and the effects of certain metal ions.

Preparation of Liver Fractions

The animals were commercial adult guinea pigs of both sexes, in normal nutrition. They were killed by a blow on the head and bled. The livers,

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as soon as they were removed, were chilled and washed in ice water. We then employed a combination of the procedures of Schneider (2) and of Hogeboom *et al.* (3) to obtain by differential centrifugation three particulate fractions and a supernatant fraction of the homogenate. Hogeboom *et al.* found that the mitochondria are thrown down as small discrete particles in 30 per cent sucrose and as clumped aggregates in isotonic saline. Accordingly we carried out the differential centrifugation in both sucrose and in two saline solutions and tested the particulate fractions obtained. The two saline solutions were KCl solution whose composition is described below and Krebs-Henseleit Ringer's solution (4).

The sucrose solution contained 300 gm. of sucrose, 0.735 gm. of L-glutamic acid, 0.203 gm. of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.174 gm. of K_2HPO_4 per liter. The KCl solution was the same as the sucrose solution except that it contained 0.9 gm. of KCl per liter instead of the sucrose. The pH of the sucrose and KCl solutions was adjusted to 7.8. The glutamic acid was added because of an impression among workers that activity with respect to coupling of oxidation and phosphorylation is better preserved in mitochondria in the presence of an oxidizable metabolite.

Diagrams 1 and 2 summarize our fractionation procedure in the sucrose and in the saline solutions.

Each fraction had the same appearance whether prepared in the sucrose or either saline solution. Sediment I was pearly white with a small amount of buff-colored material layered on top. Staining with methylene blue showed the sediment to consist mainly of nuclei; there were smaller clumps (presumably aggregated mitochondria), a few red cells, liver cells, and some débris. Sediment II was buff-colored; the staining properties of the fraction prepared with sucrose corresponded to those Hogeboom *et al.* ascribe to mitochondria. Sediment II prepared with saline solution had the same color as that prepared in the sucrose solution; approximately the same amount was obtained from a given weight of liver; the particles were much smaller than nuclei and stained faintly with methylene blue, deeply with Janus green B (3). From the centrifugal force required to throw down Sediment III the particulate matter would appear to correspond to the microsomes of Claude (6). Schneider *et al.* (7) found that most of the cytochrome *c* of liver cells appeared to be in the microsomes; Sediment III was reddish brown, quite distinct from the buff color of Sediment II, and resembled microsomes in this respect also. The supernatant fraction was a deep red transparent solution under a turbid surface layer; the fraction used was removed by careful pipetting.

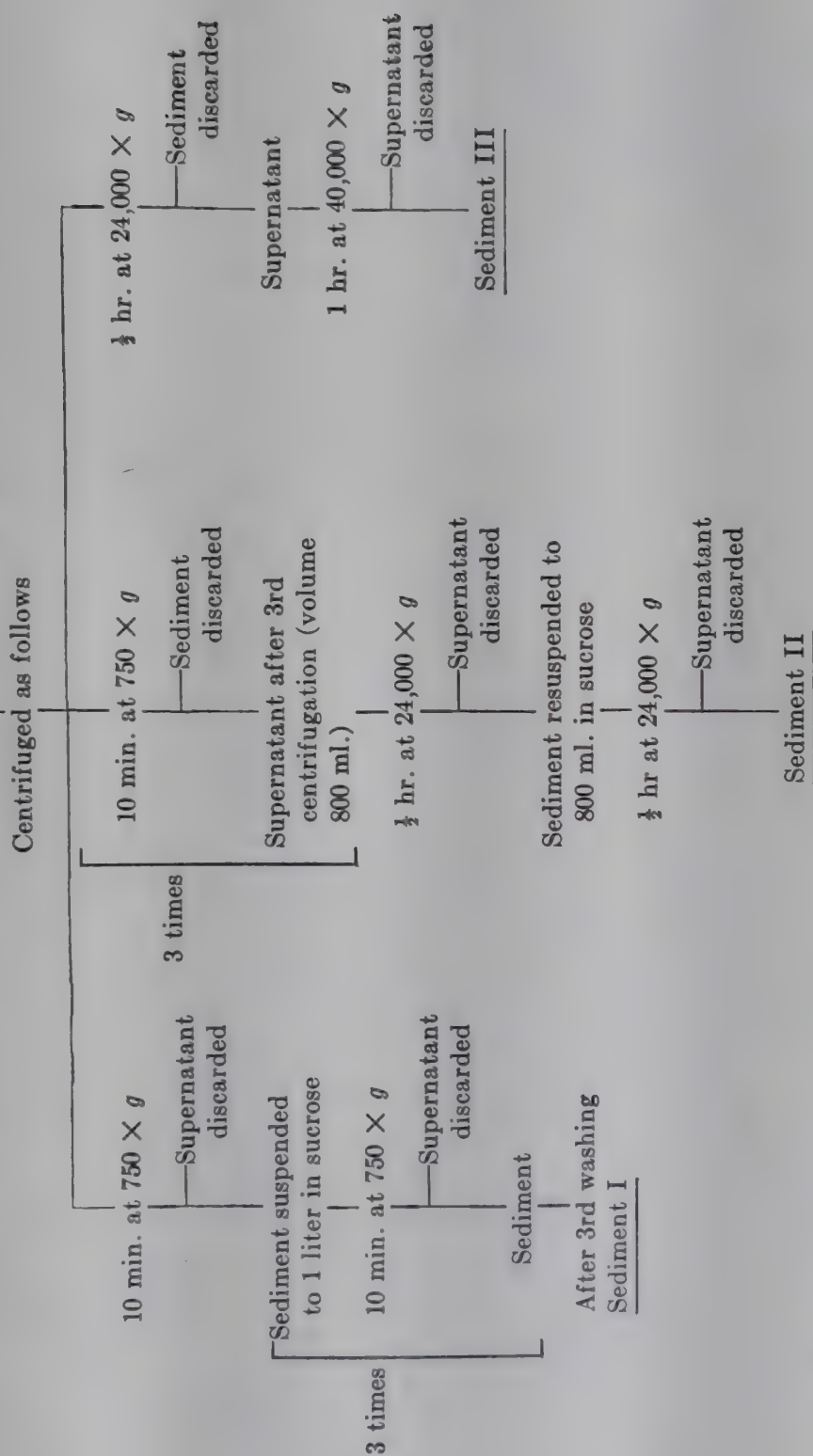
Two refrigerated centrifuges were used in the foregoing preparations. One, for centrifugal forces up to $2500 \times g$, was an International centrifuge maintained at $0-4^\circ$; the other, for higher centrifugal forces, was a Servall

DIAGRAM 1

Preparation of Fractions in Sucrose Solution

100 gm. liver + 100 ml. sucrose, Waring blender $1\frac{1}{2}$ min., pH kept above 7.3 with 1 N KOH

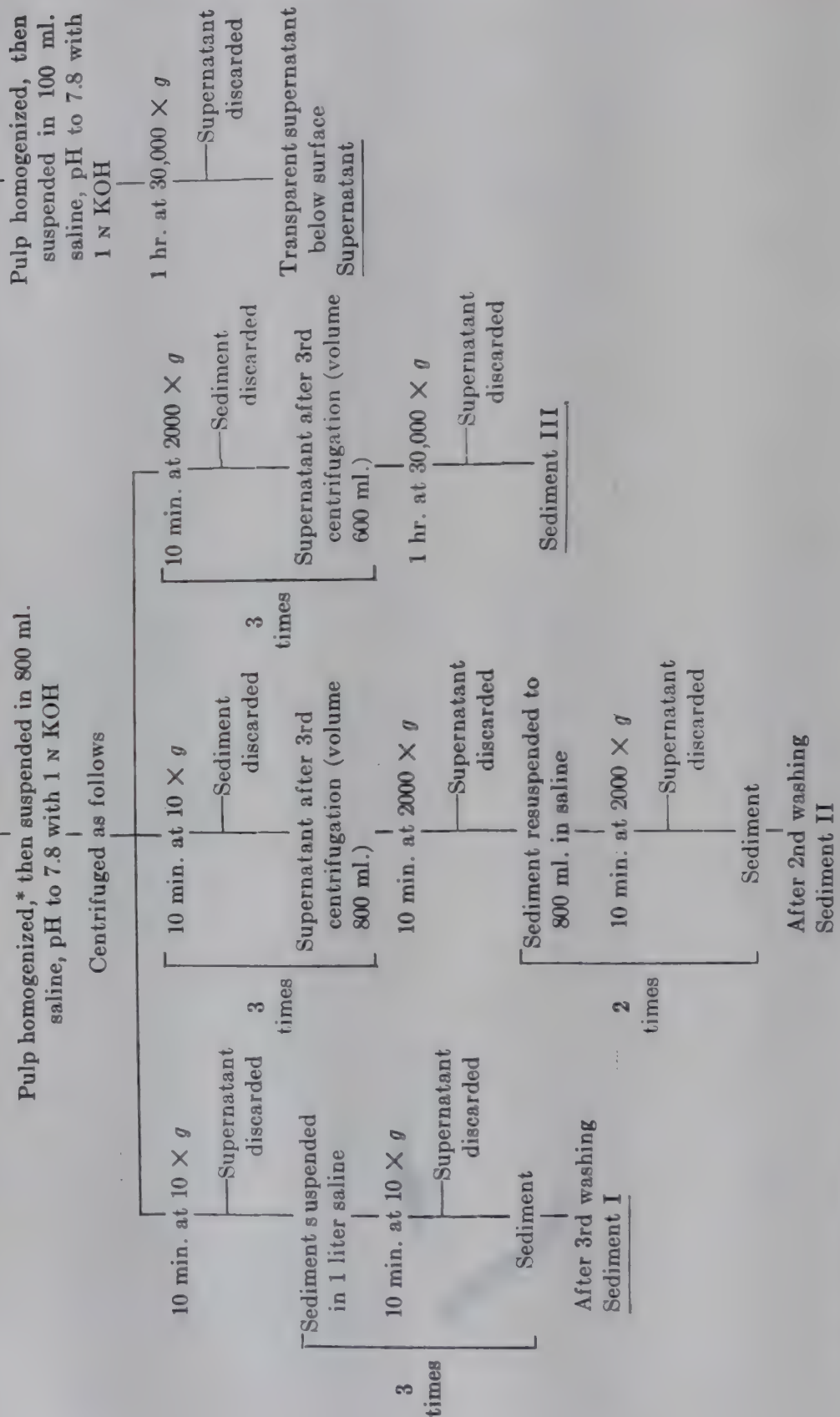
Pulp homogenized,* then suspended in 800 ml. sucrose, pH to 7.8 with 1 N KOH



* Potter and Elvehjem (5).

Preparation of Fractions in KCl Solution or in Krebs-Henseleit Finger's Solution (4)

100 gm. liver + 100 ml. saline, Waring blender $1\frac{1}{2}$ min., pH kept above 7.3 with 1 N KOH



* Potter and Elvehjem (5).

model SS-2 centrifuge; the rotor was cooled in ice before use, the bearing was kept cool with continually circulating ice water, and at the end of the centrifugation the solution was usually about 10°.

Though the fractions were not pure they exhibited functional differences and it was unnecessary for present purposes to purify them further.

The yields of the four fractions per 100 gm. of liver were approximately as follows: Sediment I 20 ml., 300 mg. of protein (dry weight); Sediment II 40 ml., 3000 mg. of protein; Sediment III 10 ml., 300 mg. of protein; supernatant fraction 150 ml., 2500 mg. of protein.

Preparation of C¹⁴-Labeled Amino Acids

Glycine—Glycine labeled in the carboxyl group was prepared by the method of Sakami *et al.* (8). It gave 55,000 c.p.m. per mg.

Synthesis and Resolution of Carboxyl-C¹⁴-DL-leucine—The Strecker synthesis with HC¹⁴N and isovaleraldehyde yields DL-leucine which was isolated as the carbobenzoxy derivative and resolved with papain and aniline. Isovaleraldehyde free from isomers was obtained by oxidation of pure leucine.

Isovaleraldehyde—10 gm. of pure leucine were dissolved in 380 ml. of water and 8.4 ml. of 4 N NaOH; the solution was cooled in ice and mixed with 76 ml. of 1 N NaOCl solution (prepared according to Raschig (9)) and 16.8 ml. of 2 N acetic acid.

The mixture was steam-distilled in the apparatus described by Langheld (10). The distillate (volume 100 to 200 ml.) was acidified with sulfuric acid. By distillation at 76–95° one obtains 4 to 5 gm. of oil with an aqueous bottom layer of about 1 gm. After shaking with solid KHCO₃, the water phase was removed, and the top layer washed three times with concentrated sodium chloride solution. The isovaleraldehyde was dried over fused CaCl₂ and distilled twice; the fraction boiling at 90–93° was collected in a receiver containing a few crystals of hydroquinone. It was redistilled immediately before use.

Carbobenzoxy-DL-leucine—The strongly alkaline solution of KC¹⁴N obtained from BaC¹⁴O₃ (11) was diluted with non-radioactive KCN to give a total of 135 mg. of KCN. The solution was acidified with 10 per cent H₂SO₄ and the HC¹⁴N distilled into a receiver kept at –20°. To the distillate (about 1 ml.) was added 0.8 ml. of concentrated ammonia while cold, then 530 mg. of freshly distilled isovaleraldehyde at room temperature. The mixture was shaken for 18 hours in a closed container. It was then transferred to a 50 ml. Erlenmeyer flask with 10 ml. of 48 per cent HBr, boiled for 30 minutes, heated on a steam bath for 4 hours, and evaporated with a stream of air at the end.

The residue was dried *in vacuo* over H₂SO₄ and KOH and boiled with 10 ml. of 1 N NaOH (glass rod to prevent bumping) until the vapor was

almost neutral to litmus. Filtration and washing with water furnished a clear colorless solution. The filtrate was acidified with 20 per cent HCl (about 0.1 ml.), concentrated to about 2 ml., and made strongly alkaline with 0.3 ml. of 4 N NaOH. After the solution was cooled in ice, the carbobenzoxylation was carried out with 0.6 ml. of carbobenzoxychloride and 0.9 ml. of 4 N NaOH in four equal portions. The alkaline reaction mixture was extracted with ether; the ether extract of the acidified mixture yielded 410 mg. of carbobenzoxy-DL-leucine. In a parallel run with non-radioactive HCN the carbobenzoxy-DL-leucine was hydrolyzed and the resulting DL-leucine isolated.

Analysis— $C_6H_{13}O_2N$. Calculated. C 54.94, H 9.99, N 10.68
 Found. " 54.81, " 10.08, " 10.86

L-Leucine—The carbobenzoxy-DL-leucine was resolved according to Bergmann and Fraenkel-Conrat (12). From 410 mg. of carbobenzoxy-DL-leucine 253 mg. of L-anilide were obtained. It was refluxed with 5 ml. of 6 N HCl for $3\frac{1}{2}$ hours, diluted with 15 ml. of water, and extracted with ether. The clear aqueous phase was evaporated to dryness *in vacuo* over H_2SO_4 and KOH.

The mixture of L-leucine hydrochloride and aniline hydrochloride was dissolved in 1.75 ml. of water. 495 mg. of non-radioactive L-leucine (Merck, methionine-free) dissolved in 2.75 ml. of 1 N HCl were added. The mixture was brought to pH 6 with concentrated ammonia and allowed to stand at room temperature for 2 hours after adding 1 volume of absolute alcohol. The leucine was filtered off and washed three times with absolute alcohol, then with ether. The yield was 461 mg. of carboxyl- C^{14} -L-leucine.

L-Leucine obtained by the foregoing procedure from carbobenzoxy-DL-leucine before dilution with non-radioactive leucine gave a rotation of $[\alpha] = +15.7^\circ$ ($C = 3.6$ in N HCl). Dunn and Rockland (13) give a value of $[\alpha]_D^{26} = +15.5^\circ$ in 21 per cent HCl.

The over-all yield of radioactive L-leucine based on the $KC^{14}N$ used was 28 per cent. The preparation used gave 27,500 c.p.m. per mg.

We have increased the yield of L-leucine by recovering the D-leucine remaining in the mother liquor of the L-anilide, racemizing it by heating in a sealed tube with $Ba(OH)_2$ at 160–170° (14), resolving, and isolating the L form as described above.

Carboxyl- C^{14} -L-lysine—Gaudry's synthesis of DL-lysine (15) provides a convenient method of preparing the amino acid labeled with C^{14} in the carboxyl group. The method was modified to adapt it to small scale work. Dihydropyran¹ was redistilled and hydrolyzed according to the method

¹ Kindly supplied by E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.

of Schniepp and Geller (16). 10 gm. (0.119 mole) of dihydropyran and 40 ml. of 0.2 N HCl were refluxed for 15 to 20 minutes; the homogeneous solution thus obtained was cooled quickly and neutralized with NaOH, and 11.4 gm. of dry $\text{Na}_2\text{S}_2\text{O}_5$ were added to it with vigorous stirring. An aliquot corresponding to 0.015 mole of dihydropyran was transferred to a flask containing 0.01 mole of NaC^{14}N . DL-Lysine dipicrate was obtained by Gaudry's procedure via the hydroxy- and bromobutylhydantoin. The dipicrate was converted to the monopicrate. The yield based on the NaC^{14}N used was 10 to 15 per cent. The monopicrate after recrystallization from water gave the following analysis.

<i>Analysis</i> — $\text{C}_{12}\text{H}_{17}\text{O}_9\text{N}_5$.	Calculated.	C 38.40, H 4.56, N 18.65
	Found.	" 38.54, " 4.68, " 18.56

The DL-lysine monopicrate was resolved enzymatically by the method used to resolve $\epsilon\text{-C}^{14}$ -lysine (17). As used the lysine gave 19,000 c.p.m. per mg.

Procedure

The reaction mixtures are given with Tables I to IV; their volume in every case was 1.01 ml. They were made up in 20 ml. Pyrex beakers and incubated at 38° in the apparatus of Dubnoff (18), under either 95 per cent O_2 and 5 per cent CO_2 or 95 per cent N_2 and 5 per cent CO_2 . At the end of the incubation the proteins were precipitated by 7 per cent trichloroacetic acid, washed, dried, and their radioactivity measured as previously described (1). The pH values given in Tables I to IV were those at the end of the incubation after the addition of 4 ml. of water.

Results

Table I gives representative results of the incorporation of L-lysine into the proteins of the four fractions in acid and alkaline pH, with and without added calcium under oxygen and under nitrogen. In the three particulate fractions more lysine was incorporated on the alkaline than on the acid side of neutrality; the addition of calcium had practically no effect; and the uptake of lysine under nitrogen was as great as under oxygen, except possibly in Sediment II where a slightly greater uptake of lysine was obtained consistently under oxygen than under nitrogen. It may be concluded, then, as a first approximation, that the three particulate fractions incorporated lysine into their proteins mainly under the "alkaline" condition. Sediments I and II were more active than Sediment III. As one obtains approximately 10 times as much of Sediment II as of Sediment I from liver homogenate, it may be said that in the whole homogenate the uptake of lysine under the "alkaline" condition occurs mostly in the particles of Sediment II.

In the supernatant fraction the uptake of lysine was greater on the acid than on the alkaline side of neutrality; there was very little without added calcium, and it was as great under nitrogen as under oxygen. The uptake in this fraction on the alkaline side of neutrality was, evidently, the same process as on the acid side, but at a less favorable pH. It may be con-

TABLE I

Incorporation of C¹⁴-Labeled L-Lysine into Proteins by Different Fractions of Guinea Pig Liver Homogenate

Homogenate fraction	pH	Cal-cium	Gas mixture	Counts per min. per mg. protein	Homogenate fraction	pH	Cal-cium	Gas mixture	Counts per min. per mg. protein
Sediment I	6.2	—	O ₂ + CO ₂	0.6	Sediment III	6.0	—	O ₂ + CO ₂	0.4
	6.2	+	" + "	1.0		6.0	+	" + "	0.5
	6.2	—	N ₂ + CO ₂	0.3		6.0	—	N ₂ + CO ₂	0.5
	6.2	+	" + "	0.7		6.0	+	" + "	0.6
	7.8	—	O ₂ + CO ₂	2.8		7.6	—	O ₂ + CO ₂	1.1
	7.8	+	" + "	4.3		7.6	+	" + "	1.2
	7.8	—	N ₂ + CO ₂	3.2		7.6	—	N ₂ + CO ₂	0.8
	7.8	+	" + "	4.2		7.6	+	" + "	1.0
" II	6.4	—	O ₂ + CO ₂	0.6	Supernatant	6.2	—	O ₂ + CO ₂	0.09
	6.4	+	" + "	0.8		6.2	+	" + "	3.45
	6.4	—	N ₂ + CO ₂	0.4		6.2	—	N ₂ + CO ₂	0.11
	6.4	+	" + "	0.5		6.2	+	" + "	3.38
	7.5	—	O ₂ + CO ₂	3.3		7.4	—	O ₂ + CO ₂	0.10
	7.5	+	" + "	3.3		7.4	+	" + "	1.8
	7.5	—	N ₂ + CO ₂	3.0		7.4	—	N ₂ + CO ₂	0.13
	7.5	+	" + "	3.0		7.4	+	" + "	1.7

All the fractions were prepared in the KCl solution. The acid reaction mixtures contained 0.7 ml. of the homogenate fraction indicated, pH adjusted to 6.2; calcium, when added, 0.01 ml. of 0.4 M CaCl₂ in 0.9 per cent KCl, adjusted to pH 6.0; 0.2 ml. of 0.1 M succinate in the KCl solution used in the preparation of the fractions, adjusted to pH 5.9; and 0.1 ml. of 0.026 M carboxyl-C¹⁴-L-lysine in the KCl solution, adjusted to pH 6.2. The alkaline reaction mixtures contained 0.7 ml. of the homogenate fraction indicated, its pH adjusted to 7.8; calcium, when added, 0.01 ml. of 0.4 M CaCl₂ in 0.9 per cent KCl adjusted to pH 6.0; 0.2 ml. of 0.2 M NaHCO₃ in the KCl solution; and 0.1 ml. of 0.26 M L-lysine-1-C¹⁴ in the KCl solution, adjusted to pH 7.5. All the reaction mixtures were incubated 2 hours.

cluded, then, that the uptake of lysine observed in the whole homogenate under the "acid calcium" condition was largely, if not entirely, confined to the supernatant fraction.

All four fractions in the experiments of Table I were prepared with the KCl solution as diluent because, even with the highest centrifugal force we could obtain, nearly 40,000 × *g*, we could not get a sufficient amount of

the supernatant fraction to work with the sucrose solution as diluent. In separate experiments we prepared the particulate fractions from the same liver, on the one hand with the sucrose solution and on the other with the KCl solution as diluent; the amount of lysine incorporated into the proteins, and the conditions with respect to pH, calcium, and independence of oxygen were the same with each fraction prepared by either method.

Table II summarizes the results on the uptake of glycine, L-leucine, and L-lysine by the three particulate fractions. The results in the pH range 7.2 to 7.7 only are given, because with glycine and leucine the uptake was less on the acid and alkaline sides of this pH range in all three fractions.

TABLE II

Incorporation of C¹⁴-Labeled Amino Acids into Proteins by Particulate Fractions of Guinea Pig Liver Homogenate

The results are expressed as millimoles $\times 10^{-3}$ per gm. of protein.

C ¹⁴ -labeled amino acid	Sediment I	Sediment II	Sediment III
Glycine.....	0.52 $\pm$ 0.07*	0.40 $\pm$ 0.09	0.075 $\pm$ 0.004*
L-Leucine.....	0.61 $\pm$ 0.15*		
L-Lysine.....	4.1 $\pm$ 1.2	3.2 $\pm$ 0.14	0.9 $\pm$ 0.05

The reaction mixtures contained 0.7 ml. of the fraction indicated, pH adjusted to 7.5; 0.01 ml. of 0.4 M CaCl₂ in 0.9 per cent KCl, adjusted to pH 6.0; 0.2 ml. of 0.2 M NaHCO₃ in either the sucrose or KCl solution according to which was used in the preparation of the fraction; and 0.1 ml. of the labeled amino acid, 0.053 M in the KCl solution, pH adjusted to 7.5. The reaction mixtures containing glycine or leucine were run for 4 hours, those with lysine for 2 hours.

* Values for preparations made with KCl solution only.

In all these experiments CaCl₂ was added to a final concentration of 0.004 M because of earlier findings that the uptake of lysine (1) and of glycine (19) was slightly augmented by this concentration of added calcium. The gas mixture used was 95 per cent O₂ + 5 per cent CO₂, as although the uptake of lysine is not significantly inhibited by anaerobiosis that of glycine is (19). The experiments with lysine were run for 2 hours and those with glycine and leucine for 4 hours, because the reaction with lysine stops at the end of 2 hours (1) while that with glycine, though much slower, continues logarithmically up to 6 hours (19).

Table II gives the averages and standard deviations of the results from eight or more different preparations of the fractions; all three amino acids were tested on the same preparation of the fraction in each experimental run. The fractions prepared with sucrose took up much less glycine and leucine than did those with the KCl solution; in fact Sediments I and III prepared with sucrose solution took up no leucine. When

the results obtained with the preparations made with sucrose solution were consistently much lower than those with KCl, the former were not included in the averages given in Table II; those averages are marked with an asterisk. We used in these experiments lysine labeled in either the carboxyl or the ϵ -carbon atom (17) and obtained essentially the same results with both.

Each of the three amino acids was taken up most actively in Sediment I and least in Sediment III. The reason for the greatest reactivity in Sediment I may be that it contained clumps of intact liver cells; cell destruction reduced the rate of incorporation of amino acids in every case which has been tested (20).

Of the three amino acids the uptake of lysine was the greatest in all three fractions. The uptake of glycine in 4 hours in Sediments I and II of 0.52 to 0.40×10^{-3} mm per gm. of protein was of the same order of magnitude as that observed by Winnick *et al.* (19) in their rat liver fraction sedimented at about $2500 \times g$ which is a composite of our Sediments I and II. They observed in their thrice washed fraction an average of 11.4 c.p.m. per mg. of protein per hour after incubation with glycine of 400,000 c.p.m. per mg. This corresponds to an uptake of 0.38×10^{-3} mm of glycine per gm. of protein per hour. Later they reported that 30 (21) to 80² per cent of the radioactivity in the protein was due to adsorbed phosphatidylserine. The radioactivity in the protein in our experiments with glycine could not have come from adsorbed phosphatidylserine formed from the labeled glycine added initially, because after treating the protein with ninhydrin the CO_2 liberated gave no counts (see Table IV below).

The uptake of leucine in Sediments II and III was irregular. In many experiments we observed no uptake; in some there was as much as 0.8×10^{-3} mm per gm. of protein. We have not found the cause of the irregular uptake of leucine in different preparations. For this reason no values are given in Table II for the uptake of leucine by Sediments II and III.

We found a synergistic action between Sediments II and III in the uptake of glycine. When the reaction mixture contained Sediments II and III, the glycine taken up varied from 120 to 250 per cent of the sum of the separate uptakes. No interaction was found between any of the other possible combinations of fractions on the uptake of glycine, nor with any combination of fractions on the uptake of leucine and lysine.

It has been found in feeding experiments that an indispensable amino acid is ineffective for growth or for recovery from protein depletion unless

² Greenberg, D. M., personal communication.

it is fed within a few hours of other necessary amino acids (22). The possibility, which these findings suggested, that the uptake of one amino acid may be increased by the presence of others was examined by comparing the uptake of labeled glycine, leucine, and lysine when the three were incubated in the reaction mixture separately and together.

Table III is a summary of the result of such an experiment. When the three labeled amino acids were added together, the protein count was the sum of the three added separately. The addition of the non-radio-

TABLE III

Incorporation of C¹⁴-Labeled Glycine, L-Leucine, and L-Lysine into Proteins When Incubated with Sediment II Separately and Together*

Labeled amino acids	Unlabeled amino acids	Counts per min. per mg. protein	
		Observed	Calculated
Glycine	Leucine + lysine	6.4	
"		6.0	
Leucine		3.0	
"	Glycine + lysine	2.5	
Lysine	Glycine + leucine	4.0	
"		4.1	
Glycine + leucine + lysine		13.1	13.4

The reaction mixtures contained 0.7 ml. of Sediment II, pH adjusted to 7.85; 0.01 ml. of 0.4 M CaCl₂ in 0.9 per cent NaCl, pH adjusted to 6.0; 0.1 ml. of each of the amino acids indicated in Krebs-Henseleit Ringer's solution, pH adjusted to 7.5. Krebs-Henseleit Ringer's solution was added when necessary to bring the final volume of the reaction mixture to 1.01 ml. The glycine, leucine, both labeled in the carboxyl group, and ϵ -C¹⁴-lysine concentrations were 4.0, 3.3, and 3.7 mg. per ml. respectively. The reaction mixtures were incubated 4 hours under 95 per cent O₂ and 5 per cent CO₂. The pH values at the end were in the range 7.3 to 7.4.

* Prepared with Krebs-Henseleit Ringer's solution (4) instead of the KCl solution as diluent.

active forms of two of the three did not affect the uptake of the radioactive amino acid added to the reaction mixture. In other experiments it was found that the uptake of each of these three labeled amino acids was the same in the presence or absence of a mixture of amino acids corresponding to the composition of casein. We may conclude, therefore, that glycine, L-leucine, and L-lysine were taken up independently under our experimental conditions.

We have surveyed the effects of added cobaltous, cupric, and manganoous salts on the uptake of glycine, leucine, and lysine by Sediment II. With final concentrations in the reaction mixture of 0.005, 0.001, 0.0005,

and 0.0001 M CuCl_2 , the uptakes of glycine, expressed as a per cent of that without added copper, were 13, 68, 93, and 100 respectively, and of lysine 33, 75, 94, and 100 respectively.

TABLE IV

Effect of Added CoCl_2 on Incorporation of Glycine and of Leucine into Proteins of Sediment II of Guinea Pig Liver Homogenate*

Concentration of added CoCl_2 in reaction mixture	Counts per min. per mg. protein			Inhibition of uptake
	Before ninhydrin treatment	In CO_2 liberated in ninhydrin treatment	Calculated re- maining in pro- tein after nin- hydrin treatment	
Glycine				
<i>M</i>				<i>per cent</i>
0	7.4	0	7.4	
0.005	9.3	6.3	3.0	59
0.001	4.1	1.8	2.3	68
0.0005	7.7	1.3	6.4	13
0.0001	8.5	0.6	7.9	0
Leucine				
0	3.0	0	3.0	
0.005	10.0	9.5	0.5	83
0.001	2.5	1.4	1.1	63
0.0005	2.6	0.7	1.9	36
0.0001	4.2	0.1	4.1	0

The reaction mixtures contained 0.7 ml. of Sediment II, pH adjusted to 7.8; 0.01 ml. of 0.4 M CaCl_2 in 0.9 per cent NaCl, pH adjusted to 6.0; 0.1 ml. of a solution of CoCl_2 in Krebs-Henseleit Ringer's solution 10 times the concentration indicated, pH adjusted to 7.0; 0.1 ml. of amino acid solution in Krebs-Henseleit Ringer's solution, pH adjusted to 7.5; and 0.1 ml. of Krebs-Henseleit Ringer's solution, or 0.2 ml. when no cobalt was added. The glycine and leucine concentrations were 4.0 and 3.3 mg. per ml. respectively. The reaction mixtures were incubated 4 hours under 95 per cent O_2 and 5 per cent CO_2 . The pH values at the end were in the range 7.3 to 7.4. In the treatment with ninhydrin 5 mg. of non-radioactive L-leucine were added to provide CO_2 as a carrier of the CO_2 from the protein. The CO_2 was trapped in $\text{Ba}(\text{OH})_2$ solution, and the BaCO_3 filtered off, dried, and counted.

* Prepared with Krebs-Henseleit Ringer's solution (4).

Added MnCl_2 over a concentration range of 0.005 to 0.0001 M was without effect on the uptake of either glycine, leucine, or lysine.

The results with added cobalt were different. The counts in the protein after exhaustive washing with trichloroacetic acid were very high, but they were also very high in the boiled controls and when the Sediment II fraction was first precipitated with acetone and dried. When the

radioactive protein was treated with the ninhydrin reagent (23) most of the counts appeared in the liberated CO_2 . Evidently the radioactive amino acids which could not be washed from the protein with trichloroacetic acid were not bound in peptide bonds. Some typical results with glycine and leucine are given in Table IV; they show that, so far as the actual incorporation of the amino acids into the proteins is concerned, CoCl_2 added to a final concentration of 0.005 to 0.0005 M was inhibitory. Similar results were obtained with L-lysine, but as it was labeled in the ϵ position, it could not serve for the measurement of the non-peptide-bound amino acid by treatment with ninhydrin.

The degree of non-peptide-bound amino acid was greater the greater the concentration of added cobalt. The amount of amino acid so bound resulting from the addition of cobalt varied with the protein; the proteins of Sediment II after precipitation with acetone were more active than the mixture of proteins of the whole homogenate; the proteins of Sediment II were more active than diaphragm proteins; the latter were more active than bone marrow cell proteins; and with egg albumin there was very little combination with the amino acids. The combination of protein with amino acid in the presence of cobalt at 38° takes about 6 hours to reach completion; it does not proceed below pH 6.0 and the rate increases progressively with increasing pH up to 8.8, which was the highest pH we examined.

The preparations of Sediment II of the experiments of Tables III and IV were made with Krebs-Henseleit Ringer's solution (4). These experiments were carried out before those of Tables I and II, in which the fractions were prepared with the KCl solution as diluent. We found no consistent differences in the incorporation of glycine, leucine, and lysine between the two saline preparations.

In controls run simultaneously with all the foregoing experiments proteins precipitated at zero time gave no counts; the proteins in liver preparations boiled for 15 minutes at pH 6.0 before addition of labeled amino acids also gave no counts; in the preparations boiled at pH 7.5 the proteins gave about 10 per cent of the counts of those in the unboiled preparations.

DISCUSSION

All four fractions of liver homogenate, three particulate fractions and the supernatant solution, incorporated labeled amino acids into their proteins, but they differed in the rate at which they incorporated each amino acid, and in the case of lysine, the three particulate fractions on the one hand and the supernatant solution on the other had different optimal conditions. These findings indicate that there are functional differences among the components of liver homogenate with regard to in-

corporation of amino acids into their proteins. There are no doubt other functional differences; the finding of Schneider *et al.* (7) that the succinoxidase activity was associated almost exclusively with the larger granules (Sediment II) points in the same direction.

Of more general interest is the conclusion which must be drawn that the incorporation of amino acids into proteins does not, in the adult cell, necessarily depend on direct participation of the nucleus. While not all the nuclei may have been removed from Sediment II, the amount left must have been small, and it is highly improbable that there were any nuclei in the Sediment III or supernatant fractions.

Labeled glycine, leucine, and lysine were incorporated into the proteins to the same extent whether each was in the reaction mixture alone or when others were added with it, radioactive or non-radioactive. It is improbable that it is the uptake of only these three labeled amino acids which has this characteristic. They were the first three amino acids tested; many, if not all, amino acids probably would behave in the same way.

Of course it is possible that other amino acids are actually necessary for the incorporation of any one into the proteins, and that, although the homogenate fraction (Sediment II) used in these experiments was washed twice, it contained, nevertheless, amino acids sufficient for the uptake of the labeled amino acids tested.

SUMMARY

1. The synthesis and resolution of L-leucine and of L-lysine labeled with C^{14} in their carboxyl groups are described.

2. Three different particulate fractions and a supernatant fraction were prepared by differential centrifugation. The four fractions exhibited functional differences in the rates at which they incorporated glycine, L-leucine, or L-lysine.

3. L-Lysine was incorporated into the proteins of the particulate fractions of guinea pig liver homogenate under one set of conditions and into those of the supernatant fraction under another set of conditions.

4. When labeled glycine, L-leucine, and L-lysine together were incubated with a homogenate fraction, the uptake, as measured by the radioactivity of the protein, was the sum of that of the three amino acids incubated separately. Addition to the reaction mixture of a mixture of non-radioactive amino acids corresponding to the composition of casein did not affect the uptake of the labeled amino acids.

5. $CuCl_2$ and $CoCl_2$ in the concentration range 0.005 to 0.0005 M inhibited the incorporation into the proteins of glycine, L-leucine, and L-lysine. The inhibition was greater the higher the concentration of cobalt.

MnCl₂ exerted neither a stimulation nor an inhibitory effect when added in the range 0.005 to 0.0001 M.

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ON THE MECHANISM OF BACTERIAL FERMENTATION OF GLUCOSE TO LACTIC ACID STUDIED WITH C¹⁴-GLUCOSE*

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This investigation was undertaken with the purpose of studying, with the use of glucose labeled with C¹⁴ in various positions, the mechanism of lactic acid formation from glucose during fermentation by a homo-fermentative lactic acid bacterium.

Rat liver glycogen, isolated 3½ hours after feeding NaHC¹³O₃, was reported by Wood *et al.* (1) to have all its detectable excess C¹³ located in positions 3 and 4 of glucose. The glucose was degraded by fermenting the C¹³-glucose to lactic acid with *Lactobacillus casei*. The lactic acid was then degraded chemically. These authors concluded that, since the labeled carbon was not randomized between positions 1 and 3 or 4 and 6 of glucose, this was proof that during the bacterial fermentation no 3-carbon symmetrical intermediate, *e.g.* dihydroxyacetone or dihydroxyacetone diphosphate, was formed. They also pointed out that carbon atoms 3 and 4 of glucose became carboxyl carbon of lactic acid. Using C¹⁴ as the tracer (2), the Western Reserve group found a trace of isotope in carbon atoms 1, 2, 5, and 6 to about 1 to 2 per cent of that found in the 2 center carbon atoms. They also reported more activity in positions 1 and 6 than in positions 2 and 5. They speculated that the formation of a 3-carbon symmetrical intermediate during animal glycolysis might be the cause of activity in carbon atoms 1, 2, 5, and 6. Glucose prepared from rat liver glycogen according to the method of Zilversmit *et al.* (3) and degraded by a *L. casei* fermentation was reported by Gibbs (4) to have 3.3 per cent of the C¹⁴ in carbon atoms 1 and 6, 2.0 per cent in atoms 2 and 5, and 94.7 per cent in atoms 3 and 4.

The experiments to be described provide (a) evidence that during fermentation with *L. casei* a partial randomization occurs between carbon atoms 1 and 3, and (b) data indicating that a change of certain conditions under which the fermentation takes place has no effect on the distribution of activity in the lactic acid.

* This research was carried out at the Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.

A preliminary report was presented at the meeting of the American Association for the Advancement of Science, New York, December, 1949.

EXPERIMENTAL

3,4-C¹⁴-Glucose—Rat liver glycogen was prepared according to the method of Zilversmit *et al.* (3). After the glycogen was isolated from the liver, it was hydrolyzed with 0.6 N H₂SO₄ for 3 hours, then passed through ion exchange columns (Amberlite IR-100-H and Duolite A-4) to insure the removal of ionic impurities. After vacuum distillation to remove water, the glucose was crystallized with absolute alcohol. This preparation was shown to be free of radioactive impurities by subjecting a portion of it to paper chromatography with (a) phenol and ammonia and (b) butanol and water saturated with propionic acid as the solvents. In each case, one band was obtained.

In order to determine whether carbon atom 1 had any activity, the 3,4-C¹⁴-glucose was converted into HCOOH (carbon atom 1) and levulinic acid by treatment with HBr, as described by Sowden (5). About 0.3 per cent of the total activity was in carbon atom 1.

1-C¹⁴-Glucose—We are indebted to Dr. J. C. Sowden for supplying us with this chemically synthesized compound. The purity of this compound was also tested with paper chromatography by use of the above solvents. This compound showed no radioactive impurities.

Uniformly Labeled Glucose—This was prepared by exposing bean leaves to C¹⁴O₂ for 24 hours.¹ Paper chromatography showed this compound to be free of radioactive impurities. This glucose had a specific activity of 1.61 mμc. per mg. of carbon.

The homofermentative *L. casei*, ATCC 7469, was used to convert glucose to lactic acid. 16 hour cultures grown in 1 per cent Bacto-peptone and 1 per cent Bacto-peptonized milk were used. The cells were washed three times with distilled water before use. The fermentations were carried out in a 2 liter desiccator under C¹²O₂, C¹⁴O₂, or air.

The reaction mixture consisted of 30 mg. of glucose, 4 per cent wet bacteria, and 1.25 mm of NaHCO₃ per mm of sugar. In Experiments 2 and 3, 1 μc. of C¹⁴ as NaHC¹⁴O₃ was added to the mixture. The final volume in each fermentation was 10 ml. A control containing the same reagents and non-radioactive sugar was run in parallel. When fermentation was complete, as judged by the absence of reducing sugar in the control (about 2 hours), the cells were centrifuged and the supernatant liquid and two water washings were poured into a modified Kutscher-Steudel extraction apparatus. The lactic acid was extracted with ether and then degraded by the method of Wood *et al.* (1). Since the lactic acid was purified by ether extractions and steam distillation only, it is possible that other organic compounds formed during the fermentation

¹ Gibbs, M., Dumrose, R., and Acher, F., unpublished data.

may react similarly to lactic acid. To determine the purity of the lactic acid, 1 μ c. of uniformly labeled glucose was fermented to lactic acid, the acid purified by ether extraction and steam distillation, and then subjected to paper chromatography. Three solvents were used: (1) phenol, (2) butanol and water saturated with propionic acid, and (3) butanol and water saturated with formic acid. One band was found in each solvent.

RESULTS AND DISCUSSION

Table I summarizes the experimental data. Fermentation 1 indicates that the lactic acid formed during the fermentation was derived entirely from the carbon atoms of glucose, since the specific activity of the carbon

TABLE I

Distribution of Radioactivity in Lactic Acid Derived by Fermentation from 1-C¹⁴- and 3,4-C¹⁴-Glucose

The C¹⁴ values are in millimicrocuries (1×10^{-3} μ c.) per mg. of carbon. In Fermentations 4, 5, and 8, an apparent activity of 1 per cent of the total, attributable to the chemical degradation (see the text), was found in the α -carbon.

Fermentation No.	Sugar	Time	Atmosphere	Carbon atom of lactic acid		
				COOH	CHOH	CH ₃
		<i>hrs.</i>				
1	Uniformly labeled	2	CO ₂	1.61	1.61	1.60
2	Unlabeled glucose	2	C ¹⁴ O ₂	0	0	0
3	" "	24	C ¹⁴ O ₂	0	0	0
4	1-C ¹⁴ -glucose	2	CO ₂	9.7	0	294
5	"	24	CO ₂	7.7	0	284
6	3,4-C ¹⁴ -glucose	2	CO ₂	361	7.7	12.2
7	"	24	CO ₂	367	7.6	13.0
8	1-C ¹⁴ -glucose	2	Air	9.0	0	290

atoms of the lactic acid was the same as the specific activity of the carbon atoms of the glucose. Since the specific activity of the carboxyl carbon was not diluted by the C¹²O₂, it appears that the organism cannot fix CO₂. This is further indicated by Fermentations 2 and 3 which show that *L. casei*, ATCC 7469, was not able to fix C¹⁴O₂. This is in agreement with the work of Slade *et al.* (6), who, using C¹³ as a tracer, reported that the homofermentative lactic acid bacteria, *Streptococcus lactis* and *Lactobacillus plantarum*, fixed no CO₂.

The data show that during the fermentation of 1-C¹⁴-glucose (Fermentation 4) about 3 per cent of the label appeared in carbon atoms 3 and 4. Since the organism was not able to fix CO₂, activity in these 2 carbon atoms cannot be explained by fixation reactions. During the fermentation

of 3,4- C^{14} -glucose, about 3 per cent of the label appeared in carbon atoms 1 and 6. Chemical degradation of the glucose indicated that carbon 1 contained only 0.3 per cent of the total activity and carbon 6 probably contained the same amount of activity. The activity in carbons 1 and 6 following fermentation is clearly greater. Thus during the fermentation, randomization occurs between carbons 1 and 3. The cause of this shift might have been the formation of a 3-carbon symmetrical intermediate, such as dihydroxyacetone or dihydroxyacetone diphosphate during the bacterial fermentation.

The chemical degradation of the lactic acid was tested by degrading² chemically synthesized $C^{14}H_3C^{13}HOHCOOH$ by the method used in this paper. No C^{13} or C^{14} was found in the CO_2 of the permanganate oxidation (carbons 3 and 4 of glucose); however, about 1 per cent of the C^{14} was found in the $HCOOH$ fraction (carbons 2 and 5 of glucose). When $CH_3CHOHC^{14}OOH$ was degraded in this laboratory, all of the activity was located in the CO_2 of the permanganate oxidation.

The apparent presence of activity in the α position of lactic acid (corresponding to carbons 2 and 5 of glucose) synthesized during fermentation of 1- C^{14} -glucose (Fermentations 4, 5, and 8) can be explained by the chemical degradation of the lactic acid. The degree of activity found in carbons 1 and 6 of the 3,4- C^{14} -glucose is not sufficient to account, by means of the chemical degradation, for the activity of carbons 2 and 5. Thus it would appear that some activity originally present in carbons 3 and 4 of the glucose appears in carbons 2 and 5 during the fermentation. It is also possible that the so called 3,4- C^{14} -glucose contained activity in carbons 2 and 5. This seems somewhat unlikely, since Topper and Hastings (7) showed that glucose synthesized in this manner and chemically degraded contained no activity in carbons 2 and 5.

It may be concluded from Fermentation 8 that the presence of oxygen had no effect on the distribution of activity. Fermentations 5 and 7 indicate that length of time of fermentation did not change the distribution of activity.

The authors wish to thank Dr. Robert Steele for his assistance during this investigation. They also wish to thank Dr. Victor Lorber for supplying us with detailed information about some of the steps of the chemical degradation of lactic acid.

SUMMARY

1. *Lactobacillus casei*, ATCC 7469, did not fix CO_2 during the fermentation of glucose to lactic acid.

Lorber, V., personal communication.

2. When 1-C¹⁴-glucose was fermented to lactic acid, 3 per cent of the label was in the COOH position of the lactic acid.
3. When 3,4-C¹⁴-glucose was fermented to lactic acid, 3 per cent of the label was in the β position of the lactic acid.
4. The randomization of 3 per cent of the activity might be caused by the formation of a symmetrical 3-carbon intermediate during the bacterial fermentation.
5. The same distribution was obtained when the fermentation was carried out under aerobic or anaerobic conditions and when the time of fermentation was increased from 2 to 24 hours.

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HYALURONIDASE INHIBITOR IN BLOOD SERUM OF SCORBUTIC GUINEA PIGS

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The chemical nature of the non-specific hyaluronidase inhibitor normally present in blood serum has not been elucidated, but quinoid and hydroquinoid compounds have been found to be most effective inhibitors of the depolymerization of hyaluronic acid by hyaluronidase *in vitro* (1). Other researches have suggested that intermediate metabolites of reduced or oxidized quinoid type may appear in blood and urine during intense salicylate therapy, alkaptonuria, and incomplete tyrosine metabolism associated with ascorbic acid deficiency (2-7). The latter condition in guinea pigs was also marked by increased serum methemoglobin and the excretion of benzoquinone acetic acid in the urine (3). Since the presence of quinoid substances in serum and urine has not been correlated with the concentration of hyaluronidase inhibitor, these measurements have been undertaken.

Methods

The non-specific hyaluronidase inhibitor was determined by the method of Dorfman, Ott, and Whitney (8) with the addition of magnesium ion to the diluted serum samples according to Freeman, Whitney, and Dorfman (9). Methemoglobin was determined by a modification of the method of Rabkin and Austin (10). Water-soluble quinones were demonstrated by Ashberg's test (3). The presence of homogentisic acid was deduced from the darkening of alkaline urine samples exposed to air. Occasional animals were sacrificed for pathological confirmation of clinical scurvy.¹ Weanling male and female guinea pigs were placed on a diet of ground rabbit chow which had been exposed to the air for 24 hours in thin layers, supplemented by 5 per cent dried yeast and a semiweekly addition of cod liver oil and wheat germ oil. On analysis, the ascorbic acid content was found to be negligible.

Blood specimens were taken by cardiac puncture from groups of

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¹ The authors are indebted to Lieutenant David Mintz, Army Institute of Pathology, for the histopathological examinations.

approximately thirty guinea pigs (weight 200 gm.) before initiation of the test diet and before, during, and after the appearance of clinical scurvy. Urine specimens were collected in 0.1 N HCl under petrolatum for 12 hour periods before and after the collection of the blood samples.

EXPERIMENTAL

Blood and urine specimens were obtained from two series of female guinea pigs while the animals were on a liberal diet of greens. After about 20 days on the deficient diet, when mild signs of scurvy appeared, the animals were bled and urine specimens were obtained. About 10 days later, when clinical scurvy had proceeded to the stage of bloody diarrhea and occasional death, blood samples were taken from the survivors.

TABLE I

Concentration of Hyaluronidase Inhibitor in Sera of Normal and Scorbutic Guinea Pigs

The results are expressed in units of hyaluronidase inhibitor as defined by Dorfman *et al.* (8).

Series	Normal diet	Deficient diet			Normal diet
	0 day	7 days	21-24 days	29-33 days	50-54 days
A, B	55		89	88	
C	65		97	87	
D	50*	70†		88	54

* After 100 mg. of ascorbic acid intramuscularly.

† After 0.25 gm. of L-tyrosine by mouth.

The data from these two groups (Table I, Series A and B) show a significant increase in the mean concentration of the hyaluronidase inhibitor with the onset of clinical scurvy, but the progression of scurvy was not accompanied by further increases in inhibitor concentration.

A comparable group of male guinea pigs (Series C, Table I) gave similar results.

Since withdrawal of ascorbic acid in Series A, B, and C, Table I, appeared to produce a rise in the inhibitor titer, the converse was examined by intramuscular injection of 100 mg. of ascorbic acid in a group of guinea pigs on a full diet of greens (Series D, Table I). The blood samples drawn 6 hours later showed no significant depression of inhibitor titer. These guinea pigs were continued on the full diet for an additional week and then placed on the deficient diet.

In view of the observations of Painter and Zilva (6) regarding the rapidity with which the biochemical defect in faulty tyrosine metabolism mani-

fested itself, an attempt was made to force the increase of inhibitor by the administration of tyrosine before clinical scurvy became evident. After 7 days on the deficient diet with no clinical evidence of scurvy, each guinea pig of Series D was given 0.25 gm. of L-tyrosine by mouth. The blood samples drawn 6 hours later showed a small rise in the mean titer of the

TABLE II

Daily Excretion of p-Quinone in Urine of Scorbutic Guinea Pig

	Days on deficient diet									
	20	21	22	23	24	25	26	27	28	29
p-Quinone, mg. %	4.2	1.26	2.52	0	0	0	0	0	4.2	0
Total quinone excreted, mg.	84	31.5	48.0	0	0	0	0	0	67	0

TABLE III

*Urinary Excretion of Quinone-Like Substances and Concentration of Hyaluronidase Inhibitor in Sera of Normal and Scorbutic Guinea Pigs (Mean Figures, Series D)**

		Normal diet	Deficient diet		Normal diet
		0 day	7 days	29 days	50 days
Blood	Hyaluronidase inhibitor units†	50	70	88	54
	Methemoglobin, mg. %	0	1.1	1.2	0
Urine	Homogentisic acid	0	+	0	0
	KI-oxidizing substance, mg. per 24 hrs.‡	0	0	7.0	0
	Methemoglobin induced by 2 cc. urine, %§	0	6.4	4.4	0

* 100 mg. of ascorbic acid were given intramuscularly at the beginning of the experiment and 0.25 gm. of L-tyrosine by mouth on the 7th day.

† Hyaluronidase inhibitor units defined by Dorfman *et al.* (8).

‡ Reported as benzoquinone acetic acid, Fishberg (3).

§ Per cent methemoglobin induced in a known amount of hemoglobin solution by addition of 2 cc. of cleared urine.

hyaluronidase inhibitor. This group then progressed to clinical scurvy. Blood and urine samples were taken from the surviving animals on the 29th day of the deficient diet, when they were immediately returned to a full diet. By the 50th day, they appeared sleek and normal in every respect and had gained weight for 10 days. The mean inhibitor concentration, last column of Table I, clearly showed a return to normal in the surviving animals that had recovered on the full diet.

While the earlier work of other investigators seems to have clearly established the presence of quinone-like compounds in blood and urine of scorbutic animals, it seemed advisable to verify the existence of similar conditions in these experiments. The daily urinary excretion of *p*-quinone is illustrated in Table II. The wide variation in daily output is similar to that reported by Fishberg (3) for human subjects.

Table III shows the appearance of quinone-like substances in the urine, the methemoglobin level, and concentration of hyaluronidase inhibitor in the serum of the animals in Series D. These data, typical of all series, seem to indicate a significant increase of quinoid-like substances in the urine at the time of maximum inhibitor titers, although direct and immediate correlation cannot be concluded.

DISCUSSION

The data demonstrate that the non-specific hyaluronidase inhibitor of guinea pig blood increased significantly as marked clinical scurvy appeared and returned to normal with recovery of the animals. Furthermore, the inhibitor level appeared to rise before the appearance of clinical scurvy if the animals were fed 0.25 mg. of tyrosine. These findings would lend considerable support to the hypothesis that compounds of the quinoid type, which are known to be active hyaluronidase inhibitors *in vitro*, may be closely related to the non-specific hyaluronidase inhibitors normally found in animal serum.

The results substantiate the findings of Fishberg (3) and Painter and Zilva (6) that the scorbutic guinea pig excretes significant amounts of methemoglobin-inducing substances and KI-oxidizing substances in the urine. The failure to demonstrate homogentisic acid excretion in any significant amounts is in agreement with the findings of Painter and Zilva.

The variation in the daily excretion of quinone-like substances by the individual guinea pig has been noted. In addition, there was variation in the magnitude of the individual response of guinea pigs to the onset of scurvy in terms of all the effects measured. However, the type of response was similar in all animals. Although no direct cause and effect relationship has been demonstrated by the data, it seems unlikely that the increase in concentration of the hyaluronidase inhibitor at the time of excretion of quinone-like substances is entirely fortuitous.

SUMMARY

1. The non-specific hyaluronidase inhibitor of normal guinea pig serum increased significantly with the onset of marked clinical scurvy and returned to normal values when the surviving animals were returned to a healthy condition.

2. Methemoglobin appeared in the blood and quinone-like compounds were excreted in the urine with the onset of scurvy and during the period of elevated level of hyaluronidase inhibitor.

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α -KETOGLUTARIC ACID OXIDASE; AN ASSAY PROCEDURE*

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A system has been described by Ochoa (1, 2) for the study of the oxidation of α -ketoglutaric acid. In that system an extract of cat heart muscle is used in a medium supplemented with magnesium and phosphate ions as well as with cytochrome *c* and adenosine triphosphate. The reaction involves both a decarboxylation and an oxidation. When the further oxidation of the product, succinic acid, is prevented by the addition of malonate, the liberation of each mole of carbon dioxide is accompanied by the uptake of 0.5 mole of oxygen.

An enzyme system was also prepared by Green *et al.* (3, 4) from pigeon breast muscle which was activated by cocarboxylase but required no other component such as magnesium ions, phosphate ions, adenosine triphosphate, or cytochrome *c*. Doubt has been expressed that these two enzyme systems are identical (4). However, it is difficult to evaluate the effect of an isolation procedure on an enzyme system.

The systems used in these studies were not specifically designed for comparing the enzyme activity of one tissue with another. In the present investigation, the oxidative decarboxylation of α -ketoglutarate has been studied further with consideration for this viewpoint. The use of the principles previously outlined in other applications of the homogenate technique has made it possible essentially to isolate the reaction rather than the enzyme system (5, 6). In this manner it is possible to oxidize α -ketoglutaric acid in a preparation of the whole tissue at what is believed to be the maximum rate. Thus the nearly impossible task of quantitatively isolating the enzyme system is avoided, as well as other difficulties associated with using fractionated tissue (7).

The result is the demonstration of another cofactor which stimulates this system and the development of an assay for α -ketoglutaric acid oxidase which is applicable to mammalian tissue.

EXPERIMENTAL

Methods—In all experiments the rate of oxygen uptake was determined by means of a conventional Warburg apparatus. The α -ketoglutaric acid determinations were made by the method of Friedemann and Haugen (8).

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A temperature of 38° and a pH range of 7.1 to 7.3 were consistently employed. The tissues were added to the Warburg flasks in the form of a 5 per cent homogenate which was prepared in isotonic potassium chloride with the Potter and Elvehjem glass homogenizer (9). Most of the work was carried on with tissues from 2 month-old Swiss (Webster strain) white mice. The amount of tissue employed and the duration of any particular experiment are given in Tables I to V. The concentration of malonate used was 0.003 M. In all experiments the whole homogenized tissue was used.

TABLE I

Optimal Conditions for α -Ketoglutaric Acid Oxidase System

The data for each component were obtained from a separate experiment with mouse liver as the enzyme source. Each material was studied at varying concentrations, while all others were maintained constant at their optimal level. The recommended level of each component is indicated by the bold faced type. In each determination 30 mg. of tissue were used. The concentration of malonate was 3×10^{-3} M.

Cytochrome <i>c</i>		Adenosine triphosphate		Phosphate		Magnesium		DPN		Coccarboxylase		Ketoglutarate	
Molarity $\times 10^5$	Q_{O_2} *	Molarity $\times 10^3$	Q_{O_2}	Molarity $\times 10^3$	Q_{O_2}	Molarity $\times 10^3$	Q_{O_2}	γ per ml.	Q_{O_2}	γ per ml.	Q_{O_2}	Molarity $\times 10^2$	Q_{O_2}
0.00	11.1	0.00	10.3	0.00	9.9	0.00	7.0	0	12.4	0.0	11.0	0.000	1.6
0.33	16.4	0.33	11.8	1.67	17.1	0.33	11.5	3.3	13.5	3.3	12.5	0.453	16.0
0.67	16.3	0.67	12.3	3.33	17.0	0.67	13.2	8.3	13.5	8.3	12.5	0.680	17.2
1.00	16.9	1.00	15.3	5.00	17.3	1.67	15.8	16.7	15.4	16.7	15.6	0.906	17.1
1.33	18.2	1.33	13.1	6.67	17.5	3.33	14.4	33.3	14.7	25.0	14.1	1.360	17.7
1.67	18.4	1.67	15.1	8.30	17.9	5.00	15.6	66.7	15.9	33.3	15.1	1.810	18.8

* Q_{O_2} values represent microliters of oxygen taken up per 10 minutes by 20 mg. of moist liver.

Materials—The cytochrome *c* and adenosine triphosphate were obtained from the Sigma Chemical Company. The Schwarz Laboratories, Inc., supplied the diphosphopyridine nucleotide, and the Bios Laboratories, Inc., supplied the thiamine pyrophosphate. Eastman Kodak furnished the malonic acid, while the α -ketoglutaric acid was prepared as described by Adkins (10).

Components of System—Part of the data of Table I confirms the requirement of the system for phosphate ions, magnesium ions, adenosine triphosphate, and cytochrome *c*, as reported by Ochoa (2). In order that a measurement of the rate of a reaction reflects the enzyme level of a tissue, it is necessary that each component be present in such concentration that the enzyme is the limiting factor determining the rate of reaction of the

system. When the optimal level of each component above was employed, it was found that the rate of reaction could be increased by the addition of thiamine pyrophosphate (cocarboxylase) and DPN (diphosphopyridine nucleotide, coenzyme I, cozymase) (Table I).

The requirement for DPN, however, was found to vary, depending upon the tissue being employed. On the addition of DPN, increases in oxygen

TABLE II

Effect of DPN on Disappearance of α -Keto Acid

The measurements were made under the optimal conditions of Table I; 100 mg. of mouse brain were used per flask; 40 mg. of mouse liver were used per flask; duration of experiment, 40 minutes. Each value represents the average of three determinations.

Tissue	With DPN		Without DPN	
	α -Keto-glutarate*	Oxygen†	α -Keto-glutarate	Oxygen
Brain.....	11.9	11.7	9.06	8.94
Liver.....	16.2	16.2	13.6	12.6

* Disappearance of α -keto acid in micromoles.

† Oxygen uptake in micromoles of 0.5 O_2 .

TABLE III

Effect of Tissue Concentration

The optimal conditions of Table I were used. The concentration of malonate was $3 \times 10^{-3}\text{ M}$.

	Liver per flask				
	20 mg.	30 mg.	40 mg.	50 mg.	60 mg.
O_2 uptake per 10 min., μl	15.0	24.7	35.1	48.5	56
" " " 20 mg. tissue per 10 min., μl ..	15.0	16.4	17.6	19.6	18.6
α -Keto acid disappearance per 40 min., mg ..	1.04	1.55	2.14	2.86	3.04
" " " " 20 mg. per 40 min., mg	1.04	1.03	1.07	1.14	1.01

uptake obtained with brain and liver were 32 and 27 per cent, respectively, while in heart a stimulation of less than 4 per cent was shown. The effect of DPN on the α -keto acid disappearance closely paralleled the increases in oxygen uptake (Table II).

On the addition of malonate in the range of 0.001 to 0.008 M, the rate of disappearance of α -keto acid was essentially unaffected but, at low levels of this range of malonate concentrations, in some experiments, the oxygen uptake was reduced. An excess of malonate was avoided because

it forms a complex with magnesium ions and would possibly inhibit the α -keto acid oxidation (11).

If the enzyme concentration is the limiting factor in the system, then the rate of reaction as measured by the oxygen uptake or by the disappearance of α -ketoglutaric acid must be proportional to the amount of homogenate added. Studies with various levels of tissue demonstrate the linear relationship in this system of the α -keto acid disappearance and the oxygen uptake to the amount of homogenate present (Table III).

TABLE IV
Relation of Oxygen Uptake Time

The optimal conditions of Table I were used and the concentration of malonate was 3×10^{-3} M.

	10 min.	20 min.	30 min.	40 min.	50 min.
	μ l.	μ l.	μ l.	μ l.	μ l.
O ₂ taken up.....	22.0	49.2	75.5	102.1	126.6
" " " in 10 min. by 20 mg. of tissue	14.7	16.4	16.6	16.3	16.6

TABLE V
 α -Ketoglutaric Acid Oxidase of Mouse Tissue

The measurements were made on the homogenate of the whole tissue added at convenient levels to the flasks. The data are expressed in microliters of oxygen per 20 mg. of moist tissue per 10 minutes.

Tissue	No. of samples	Oxygen uptake
Liver.....	6	17.0 $\pm$ 3.0
Heart.....	4	31.5 $\pm$ 4.0
Kidney.....	2	18.5 $\pm$ 2.0
Brain.....	3	6.0 $\pm$ 2.0
Spleen.....	4	2.4 $\pm$ 1.0
Lung.....	3	0.6-0 $\pm$ 1.0

The stability of the system is demonstrated in Table IV. These data indicate that the total oxygen uptake is proportional within limits to the duration of the experiment. When liver was used, the rate of oxygen uptake was constant up to 50 minutes; however, the stability of the system varies with the tissue used. The activity of brain preparations often declines after 20 minutes. In most oxygen determinations, the first two 10 minute readings were averaged.

Assay Results—This assay technique was applied to a survey of various tissues of the mouse. The values obtained from a number of determina

tions are presented in Table V. Of those tissues tested, heart was the most active. Liver and kidney were about equally active and in each case less active than heart muscle. The activity of brain and spleen was considerably lower than liver, while lung was almost inactive.

DISCUSSION

The oxygen uptake of the system described is believed to be a measure of the rate at which various tissues oxidize α -ketoglutaric acid. This is indicated by the close parallel between the oxygen and α -keto acid determinations.

The additions of cytochrome *c* are such that further additions do not stimulate the system and the adequacy of the hydrogen transport system with regard to cytochrome oxidase has been previously established (12). In the oxidation of malic acid, Q_{O_2} values of 100 are obtained in mouse liver without a limiting action of the cytochrome oxidase.

The ability of DPN to function in this system, as indicated by the stimulation of the α -ketoglutaric acid oxidase activity of brain and liver, was suggested by other studies with the glutamic dehydrogenase system. Von Euler *et al.* reported that α -iminoglutaric acid could be reduced to glutamic acid in the presence of reduced DPN (13). Later Krebs and Cohen (14) reported that the reduction of α -iminoglutaric acid could be coupled with the oxidation of α -ketoglutaric acid. This suggested that DPN might act as a hydrogen acceptor in the oxidation of α -ketoglutarate. The increase in the rate of disappearance of α -ketoglutaric acid on the addition of DPN indicates further the very close relation between the decarboxylation and oxidative part of the reaction.

The difference in DPN requirement for various tissues may be a reflection of the initial tissue content or the result of differing levels of DPNase activity (15, 16). These differences in response do, however, indicate the importance of adding DPN to the system, since its presence alters the activity of these tissues relative to one another.

SUMMARY

1. Diphosphopyridine nucleotide has been found to be required for the oxidation of α -ketoglutaric acid by liver and brain homogenates.
2. A method for the assay of α -ketoglutaric acid oxidase activity of mammalian tissues was developed.
3. A survey was made of the α -ketoglutaric acid oxidase activity of various mouse organs. The activities of these tissues were found to vary in the following decreasing order: heart, kidney, liver, brain, spleen, and lung.

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THE ANEMIA OF INFECTION

X. THE EFFECT OF INFECTION ON THE ABSORPTION AND STORAGE OF IRON BY THE RAT*

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Studies in this laboratory (1-4) have shown that in the presence of inflammation there is a disturbance in iron metabolism as manifested by hypoferremia and a decrease in the concentration of the metal-binding protein in the serum. When iron is given by mouth, little change takes place in the levels of plasma iron (flat, oral iron absorption curve), and even following the intravenous injection of iron there is but a transient rise in the plasma iron. These findings have been interpreted as indicating that diversion of iron from the plasma occurs in inflammation. The anemia itself, however, is not simply the consequence of lack of iron, for the intravenous administration of larger amounts of iron fails to relieve it (1, 5-8).

Menkin and Menkin (9, 10) have shown that iron accumulates in the inflamed area where, they postulate, it may exert a beneficial function. This, however, does not account for the disturbance in iron metabolism which is associated with inflammation, for it has been demonstrated in this laboratory that the greatest proportion of radioactive iron, injected intravenously into animals in which inflammation has been produced experimentally, is diverted primarily to the liver and spleen, rather than to the inflamed tissues (11).

The questions may be asked, does the iron which is diverted to the liver and spleen perform a special function there or does it simply represent iron which is stored because hemoglobin formation is diminished? If the first were true, it would be logical to expect that the need of the body for iron during inflammation might be increased and that, as a consequence, the absorption of iron from the intestinal tract might be augmented. If the second were true, it would be expected that the body would already possess more iron than is needed and, as a result, less might be absorbed. The purpose of the experiments to be described was to study the influence of inflammation on the absorption of iron from the gastrointestinal tract.

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Studies of the iron content of the liver and spleen of patients dying of various infections and malignancies have in general indicated an increase in total splenic iron but have revealed no consistent change in liver iron (12-14). Such studies, however, do not indicate accurately the amount of iron absorbed, since they do not take into account the increase in tissue iron which, if loss of blood has not occurred, results from anemia and a decrease in the amount of total circulating hemoglobin.

Schaefer (7, 8, 15) carried out studies on iron balance in children with infection and made measurements of tissue iron in mice with experimentally induced infections. He concluded that during the febrile period the absorption of iron from the gastrointestinal tract is increased. It is difficult, however, to accept his data because of the small number of mice used in his experiments, the many technical difficulties accompanying balance studies, and the extremely small differences which he observed in the iron content of the tissues of the various groups.

Hahn, Bale, and Whipple (16) administered radioactive iron orally to several dogs before and after turpentine injections. Because the uptake of radioiron by the red blood cells was decreased, they concluded that iron absorption is reduced in the presence of inflammation. Such a conclusion was not justified, however, for uptake of radioiron may also be reduced when iron absorption is unchanged but hemoglobin synthesis is reduced (17, 18). In studies with injected radioactive iron it was shown in this laboratory that in the presence of inflammation the rate of hemoglobin synthesis is impaired (18).

In order to ascertain the amount of iron actually absorbed over a given period of time it is necessary to determine the total iron content of the body and to compare this value with suitable controls. This can be done most accurately by feeding a portion of the iron in a radioactive form and then, after a given time, determining the total amount of the labeled iron in the body. Several of the experiments reported here have been carried out in this way.

Methods

Animals—A total of 236 male rats of the Sprague-Dawley strain was used in this study. The animals in Experiments I, II, and III were fed a diet consisting of purified casein 18 per cent, sucrose 66 per cent, hydrogenated vegetable oil (Snowdrift) 6 per cent, salt mixture¹ without added iron 4 per cent, brewers' yeast 4 per cent, and cod liver oil 2 per cent. Vitamin E was supplied as mixed tocopherols in the amount of 4 mg. per

¹ The salt mixture had the following percentage composition: NaHCO₃ 2.74, CaHPO₄ 25.0, MgSO₄ 14.0, K₂CO₃ 4.65, KCl 31.2, CuSO₄ 0.24, MnSO₄ 0.15, KI 0.15, and NaF 0.03.

rat per week, added to the diet. The food intake of the control non-infected groups was restricted in an effort to keep their growth rate about equal to that of the animals in which an infection was produced.

The animals in Experiment IV were fed Purina dog chow.

Infection—Infections were maintained in the animals by injecting into a knee joint either 0.5 ml. of an 18 hour broth culture of *Staphylococcus aureus* or 0.5 to 1.0 ml. of an 18 hour brain-heart culture of mixed organisms obtained by swabbing out the mouths of the rats. Injections were given at varying intervals, depending upon the appearance of the animal. Usually a single injection resulted in a severe cellulitis of the entire leg, which lasted 12 to 15 days. Hemorrhage occurred at the site of injection in a few of the animals. These were discarded from the experiment.

Digestion of Whole Rat—The carcass of each rat was placed in a 1000 ml. beaker and 100 ml. of concentrated nitric acid and 25 to 30 ml. of distilled water were added. The beaker was allowed to stand in an oven at 37° until the rat had completely dissolved except for a few hairs. The solution was then boiled gently until the volume had been reduced to about 125 ml., at which time a clear to slightly cloudy solution was obtained. This was cooled to room temperature and the layer of fatty material was then skimmed off the top and set aside. The solution was transferred quantitatively to a 200 ml. volumetric flask and made up to volume with washings (double distilled water) from the beaker and the fat. By this method it was possible to obtain 100 per cent recovery of injected radioactive iron.

Determination of Radioactive Iron—For the determination of radioactive iron a modification of the method of Vosburgh *et al.* (19) was used. 50 ml. aliquots of the above whole rat digests along with one-fourth of the fatty material were transferred to a 200 ml. Vitreosil (silica) evaporating dish. To avoid spattering, the nitric acid was carefully removed by evaporation at around 80–100° and then dry-ashed by placing the dish in a cool muffle furnace and slowly increasing the temperature to 600°. The material was allowed to ash overnight at this temperature.

The resulting ash was dissolved in two to three successive 30 ml. portions of 6 N HCl by warming on a sand or steam bath. The combined HCl solutions were transferred to a pear-shaped separatory funnel of suitable size and concentrated HCl was added to make the concentration close to 8 N. This seems to be the optimum normality of HCl for most rapid extraction. The iron was then extracted from the HCl solution by vigorous shaking with two 20 ml. portions of isopropyl ether, followed by 10 ml. portions until extraction was complete as evidenced by a negative test for iron in the residual solution when thioglycolic acid and α, α' -dipyridyl were added to a small sample and the solution made basic to

Congo red with NH_4OH . Usually two 20 ml. portions and one 10 ml. portion sufficed.

The combined isopropyl ether extracts were then shaken vigorously with 15 ml. of saturated ammonium oxalate solution to extract the iron. This was repeated with 7 to 8 ml. portions until either the extract or the isopropyl ether gave a negative test for iron. Usually three extractions were sufficient.

The combined ammonium oxalate extracts were then filtered into a previously prepared electroplating cell, as described by Greenberg *et al.* (20), and the filter paper was washed with the saturated ammonium oxalate solution. Enough inert FeCl_3 solution was added to each cell to give a total of 5 mg. of iron to be plated. This was followed by about 70 mg. of powdered ascorbic acid and a drop of phenol red indicator, after which concentrated NH_4OH was added until the solution turned violet. Each time samples were plated pilots were prepared by transferring 1 ml. aliquots of a 1:100 dilution of the radioactive stock solution to the plating cell and adding 20 to 25 ml. of saturated ammonium oxalate solution, followed by carrier iron, indicator, etc., as for the samples.

Plating was carried out at 400 to 600 ma. for several hours or until the solution gave a negative or only a very faint test for iron after standing 20 to 30 minutes to allow for full color development owing to the large amount of oxalate.

The plates were removed, dried, and covered with a thin layer of oil in benzene (1:100), and then counted, with a model 2 Radiation Counter Laboratories mark 1 Geiger tube with a thin mica window (2 to 3 mg. per sq. cm.). This counts chiefly the β -radiations from the Fe^{59} . Each plate was counted for two or more 4 minute intervals.

Determination of Total Body Iron—The entire rat body was digested as described above and 5 ml. aliquots of the nitric acid solution were dry-ashed in the same manner as were the aliquots for radioiron. The ash was then taken up in three successive 10 ml. portions of 6 N HCl and made to volume in 50 ml. volumetric flasks. The iron was determined by using 2 ml. aliquots as described in an earlier paper (21).

Determination of Blood, Liver, Spleen, and Carcass Iron—The animals were viviperfused by the method of Copp and Greenberg (22), except that the perfusion fluid was given through the femoral vein rather than through the abdominal vena cava. The entire perfusate was collected, the volume measured, and hemoglobin and iron determined on suitable aliquots.

The livers and spleens were removed, superficially dried with gauze, and weighed. Suitable portions of these organs were then digested in 2 ml. of concentrated H_2SO_4 and successive small portions (6 to 18 drops)

of nitric acid added as required for clearing. These digests were transferred to 25 ml. volumetric flasks and made to volume with double distilled water. Iron was determined on suitable aliquots with use of α, α' -dipyridyl as described previously (21), except that, because of the greater acidity of the samples, saturated sodium acetate instead of acetate buffer was used to make the pH basic to Congo red. The rat carcasses were dry-ashed and the iron determined as described previously (21).

EXPERIMENTAL

Experiment I—A total of forty-two rats was used in this experiment. An infection was maintained in thirty-three of these, but only thirteen survived a sufficient length of time to be satisfactory for the experiment. All of the animals received 0.5 ml. of an iron solution, containing 2 mg. of

TABLE I
Experiment I. Retention of Radioactive Iron in Normal and Infected Rats

	No. of rats	Body weight	Hb	Total iron		Radioactive iron	Total radioiron absorbed
		gm.	gm. per cent	mg. per rat	mg. per 100 gm. rat	c.p.m. per 100 gm.	per cent
Control.....	9	91.4 ±2.0	18.1 ±1.12	7.10 ±1.70	7.98 ±1.90	2080 ±470	22.3
Infected.....	13	90.8 ±6.2	17.6 ±1.75	6.11 ±0.87	6.70 ±0.98	1431 ±242	15.3

The figures represent the mean $\pm$ the standard deviation.

total iron and 1.51×10^3 countable counts per minute of radioactive iron, every other day for 10 days by stomach tube. The iron solution was made up as follows: 1 unit of radioactive iron² was dissolved in 80 ml. of concentrated HCl and made up to 120 ml. with water. 2 ml. of this original solution were diluted to 100 ml. with 0.1 N HCl for the stock solution. To 2 ml. of the radioactive stock solution, 34 mg. of natural $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ were added and the final volume was made up to 25 ml. with distilled water. The iron solutions were made up fresh each time prior to administration. A period of 4 days was allowed after the last iron administration for elimination of the non-absorbed iron from the gut. At this time, 15 days after the initiation of the infection, the animals were sacrificed.

The results are summarized in Table I. No anemia was observed in

² The radioactive iron was a mixture of Fe^{55} and Fe^{59} obtained from the Clinton Laboratories as irradiated units in the form of powdered iron. Each unit contained approximately 1.0 mc. of Fe^{59} and 0.9 mc. of Fe^{55} in 17 gm. of iron target material.

this period of time in the infected group. The total iron, whether expressed as mg. per rat or per 100 gm. of body weight, was lower in the infected rats than in the controls, but this difference was not significant ($t = 2.02$) by Fisher's t test (23). On the other hand, the total radioactive iron of the body was significantly lower ($t = 4.07$) in the infected group compared with the control group.

Experiment II—A total of 120 rats was used in this experiment. All the animals received 2 mg. of iron as ferrous chloride orally every other day. The experiment was divided into two groups, A and B. In approximately one-half of the animals in each of the groups an infection was produced; the other half served as controls. The animals in Group A were sacrificed 0, 4, 8, 12, and 16 days after the induction of infection and the total body iron was determined. The animals in Group B were sacrificed by viviperfusion at the same intervals of time, and the iron content of the liver, spleen, blood, and carcass was determined. All surviving animals received 2 mg. of iron as ferrous chloride by stomach tube on 0, 1, 4, 5, 8, 9, 12, and 13 days, except, those animals that were being sacrificed on 0, 4, 8, and 12 days. These were given no iron on the day they were sacrificed. This schedule of iron administration allowed a 3 day period after the last injection for the iron to be eliminated from the gastrointestinal tract before the animals were sacrificed. A severe localized cellulitis was maintained throughout the first 12 days. From the 12th to 16th day some of the infections were beginning to subside.

The results for Group A (total body iron) are shown in Table II. The total body iron in the control non-infected animals was greater than in the infected animals. When the differences between the means were analyzed statistically for significance by Fisher's t test (23), the differences in iron gained at 4 days and 16 days after the induction of the infection were not found to be significant, while those at 8 and 12 days were significant even at the 1 per cent level. This is readily explained by the fact that the effects of the infection were not fully manifest at 4 days, whereas following the 12th day considerable improvement had taken place.

The data for the distribution of iron in the viviperfused animals, Group B, are presented in Table III. There was no significant difference between the control and infected animals with respect to spleen, carcass, or total iron. The liver iron was significantly decreased in the infected animals at 4, 8, and 12 days, compared to the corresponding control animals.

Experiment III—A total of thirty-six rats was used in this experiment. Ten animals served as non-infected controls. Twenty-six animals were given an infection. Of these, only ten were surviving on the 27th day, at which time the experiment was terminated by viviperfusion.

The results are summarized in Table IV. As in Experiment II, the

livers of the infected rats contained significantly less iron than the livers of the control animals. The infected animals had a mild but statistically significant anemia. The spleen and carcass iron was essentially the same in both groups.

Experiment IV—Unpublished work from this laboratory, as well as studies which have appeared recently (24–26), indicate that the type of diet profoundly influences iron absorption. Diets high in calcium and phosphorus tend to decrease the absorption of iron. As a result of these findings it seemed worth while to study the influence of infection on the

TABLE II

Experiment II, Group A. Total Body Iron in Control and Infected Rats Determined at Various Intervals Following Onset of Infection

	Sub-group	Time following <i>Staphylococcus</i> injection				
		0 day	4 days	8 days	12 days	16 days
No. of rats	C. I	8	6 8	4 8	4 5	5 4
Body weight, gm.	C.	80.2	79.8	90.5	103.0	94.6
		± 8.2	± 16.3	± 12.0	± 7.1	± 2.1
	I.		75.0	88.0	86.0	88.5
Total iron, mg. per rat			± 7.8	± 11.1	± 7.2	± 1.3
	C.	3.69	4.63	6.14	8.18	6.96
		± 0.41	± 0.78	± 0.48	± 1.97	± 0.96
Iron gained per 100 gm., mg.	I.		4.26	4.89	4.81	5.62
			± 0.39	± 0.76	± 0.49	± 0.65
	C.	0	1.30	2.24	3.22	2.74
			± 1.09	± 0.82	± 1.28	± 1.22
	I.		1.07	0.93	0.97	1.70
			± 0.52	± 0.33	± 0.15	± 0.65

C., control; I., infected. Means $\pm$ standard deviation.

absorption of iron from food sources rather than in the form of readily available inorganic iron salts.

A total of thirty-eight animals was used in this experiment. The diet consisted entirely of Purina dog chow which was fed *ad libitum*. The animals were divided into two groups, A and B. In fourteen of the nineteen animals in Group A an infection was produced and all of the animals surviving on the 27th day were sacrificed and total body iron was determined. Likewise, in fourteen of the nineteen animals in Group B an infection was produced, and all of the animals surviving on the 27th day were sacrificed by viviperfusion. In these the iron content of the liver, spleen, blood, and carcass was determined.

The data for Group A are summarized in Table V. The total body

iron was less in the animals in which an infection was produced than in the controls, but this difference was not found to be statistically significant.

TABLE III

Experiment II, Group B. Distribution of Iron in Control and Infected Rats Viviperfused at Various Intervals Following Injection of Staphylococcus aureus

	Sub-group	Time following <i>Staphylococcus</i> injection				
		0 day	4 days	8 days	12 days	16 days
No. of rats	C. I.	9 8	8 8	5 7	4 4	4
Body weight, gm.	C.	74.8 ±10.4	83.5 ±11.2	89.4 ±9.7	101.5 ±7.2	
	I.		74.9 ±10.2	87.5 ±10.3	89.8 ±12.3	99.0 ±12.8
Liver iron, mg. per rat	C.	0.17 ±0.06	0.26 ±0.06	0.31 ±0.06	0.44 ±0.09	
	I.		0.18 ±0.04	0.24 ±0.05	0.27 ±0.04	0.26 ±0.10
Spleen iron, mg. per rat	C.	0.039 ±0.012	0.048 ±0.015	0.069 ±0.021	0.049 ±0.022	
	I.		0.057 ±0.018	0.049 ±0.012	0.052 ±0.030	0.72 ±0.007
Blood iron, mg. per rat	C.	1.96 ±0.31	2.33 ±0.33	2.66 ±0.21	2.84 ±0.64	
	I.		2.17 ±0.32	2.56 ±0.41	2.78 ±0.32	3.37 ±0.32
Carcass iron, mg. per rat	C.	0.76 ±0.18	0.98 ±0.18	0.96 ±0.08	1.28 ±0.09	
	I.		0.92 ±0.13	1.09 ±0.15	1.13 ±0.07	1.37 ±0.19
Total iron, mg. per rat	C.	3.94 ±0.46	3.61 ±0.51	4.01 ±0.34	4.60 ±0.64	
	I.		3.32 ±0.45	3.94 ±0.51	4.23 ±0.36	5.08 ±0.53

Means ± standard deviation.

The data for Group B are summarized in Table VI. There was no significant difference in the iron content of the liver, blood, or carcass of the infected animals compared with the controls. However, the spleens of the infected group showed a significant increase in iron content.

It should be pointed out that the values for the total body iron per unit weight of all of the rats in this experiment were considerably lower than the corresponding values in Experiments I, II, and III. This is even

more striking when one considers that in the first three experiments the rats received only 1 mg. of iron per day, while the rats on the dog chow diet received more than 3.25 mg. of iron per day.

TABLE IV
Experiment III. Distribution of Iron Following Viviperfusion in Control and Infected Rats

Group (10 rats each)	Body weight	Hb	Liver iron	Spleen iron	Carcass iron
	gm.	gm. per cent	mg.	mg.	mg.
Control.....	146	17.8	0.49	0.109	3.08
	±17	±0.53	±0.20	±0.06	±0.37
Infected.....	133	15.1	0.27	0.121	2.88
	±11	±1.03	±0.14	±0.06	±0.56

Means ± standard deviation.

TABLE V
Experiment IV, Group A. Total Body Iron in Control and Infected Rats Fed Dog Chow

Subgroup	No. of rats	Body weight	Hb	Total iron	
		gm.	gm. per cent	mg. per rat	mg. per 100 gm.
Control.....	5	128 ± 6	17.2 ± 0.5	7.35 ± 0.87	5.73 ± 0.54
Infected.....	8	110 ± 14	16.3 ± 0.5	6.47 ± 0.78	5.90 ± 0.36

Means ± standard deviation.

TABLE VI
Experiment IV, Group B. Iron Distribution Following Viviperfusion in Control and Infected Rats Fed Dog Chow

Subgroup	No. of rats	Body weight	Hb	Liver iron	Spleen iron	Blood iron	Carcass iron	Total iron	
		gm.	gm. per cent	mg.	mg.	mg.	mg.	mg. per rat	mg. per 100 gm.
Control.....	5	147	18.4	0.59	0.051	4.19	2.28	7.154	4.86
		±18	±0.2	±0.07	±0.013	±0.36	±0.23	±0.64	±0.58
Infected.....	7	132	16.0	0.58	0.090	4.15	2.23	6.981	5.34
		±15	±1.4	±0.08	±0.024	±0.46	±0.22	±0.11	±0.57

Means ± standard deviation.

DISCUSSION

It is evident that under the conditions of Experiments I, II, and III infection led to a decrease in the absorption of iron from the gastrointestinal tract and as a result of this there was a significant decrease in

the amount of iron in the liver. No evidence has been found that during infection there is an increase in iron absorption, as Schaefer (7, 8, 15) reported.

Decreased absorption of iron, along with a rapid rate of removal of the metal from the plasma, helps to explain the low rise in plasma iron which we have observed in patients with infections following the oral administration of iron (1). It is unlikely, however, that decreased absorption of iron from the gastrointestinal tract plays a significant rôle either in the production of the hypoferremia which accompanies infections or in the anemia associated with infections. Hypoferremia may occur as soon as 24 or 48 hours after the onset of an infection (27) and is only temporarily alleviated by the parenteral administration of massive doses of iron.³ Likewise, the anemia is not relieved by the parenteral administration of large doses of iron (1).³

Since the anemia is not relieved by iron, it must be postulated that infection in some other manner results in impaired hemoglobin synthesis. Furthermore, decreased hemoglobin synthesis *per se* does not explain the alterations in iron metabolism, since in such conditions as pernicious anemia, aplastic anemia, Mediterranean anemia, and anemia due to protein deficiency hypoferremia does not occur (27, 28). The hypoferremia and other manifestations of disturbances in iron metabolism, such as decreased absorption of iron, rapid removal of iron from the serum, and a decrease in the concentration of the metal-binding protein in the serum, are probably the consequence of other reactions associated with inflammation. As is discussed elsewhere, one of these reactions is concerned with the activity of the reticulo-endothelial system.

Several European investigators (29) have suggested that iron may in some way function in the defense mechanism of the body against infection. It might be postulated that by decreasing the absorption of iron, by decreasing the iron-carrying capacity of the serum, and by rapidly removing iron from the serum the infected organism takes every step possible to keep the plasma iron at a low level. The reason for this is not evident nor is it obvious whether this alteration in iron metabolism serves a beneficial function or is harmful. Since during infections the general over-all metabolism of the tissues is increased, and since iron porphyrin enzymes other than hemoglobin are concerned with cellular respiration, it is possible that the need for iron by the tissues is increased. However, if this were true, it is strange that the absorption of iron is not increased and that the parenteral administration of large doses of iron cannot satisfy this demand and eventually alleviate the hypoferremia.

Finally, it must be considered that, although in these experiments

³ Unpublished observations.

infection resulted in a decrease in the absorption of iron, infections in human subjects may not result in such a decrease. The amount of iron absorbed is obviously dependent upon many factors such as the type of diet, the amount and type of iron, gastric acidity, the presence of reducing substances in the diet, and other unknown factors. Furthermore, different types, locations, and severities of infection may differ widely in their effects on iron absorption and metabolism.

SUMMARY

The influence of experimentally induced infections on the absorption of iron has been investigated in rats by determining the total amount of both non-radioactive and radioactive iron retained in the body during the period of infection. In the presence of infection, a decrease was observed in the amount of iron retained.

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THE ANEMIA OF INFECTION

XI. THE EFFECT OF TURPENTINE AND COBALT ON THE ABSORPTION OF IRON BY THE RAT*

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In a previous report (1) from this laboratory it was demonstrated that by the simultaneous administration of cobalt the anemia associated with inflammation, as produced by the injection of turpentine, was not only prevented from developing but polycythemia was produced instead. In Paper X of this series (2) it was demonstrated that the absorption of iron from the gastrointestinal tract was decreased in rats in which a bacterial infection was produced. The purpose of this paper is to determine whether sterile (turpentine) abscesses have a similar effect on iron absorption, and, if so, whether the simultaneous administration of cobalt can reverse this effect. Theoretically, the iron incorporated into the newly synthesized hemoglobin of cobalt-treated, turpentine-injected, polycythemic rats could come from either tissue stores or the gastrointestinal tract or from both sources.

EXPERIMENTAL

Animals—A total of 57 weanling male Sprague-Dawley rats was used in this study. The animals were fed the purified diet, without added iron, described in Paper X (2). All animals received 0.5 ml. of iron solution, prepared as described previously (2), containing 2 mg. of total iron as $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 622 countable counts of radioactive iron per minute by stomach tube 24, 48, and 96 hours after each turpentine injection. Each rat received a total of thirteen such iron feedings, representing a total of 8086 countable counts per minute of radioactivity. 5 days were allowed to elapse between the last iron feeding and sacrificing the animals. All animals were killed by ether anesthesia 32 days after the first turpentine injection.

Nine rats were used as controls and were given neither turpentine nor cobalt. In eighteen rats 0.25 ml. of rectified oil of turpentine (Rexall) was injected each week intramuscularly into the hind leg. Twelve rats

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were given 0.125 mg. of cobalt daily as cobaltous chloride in an aqueous solution, injected intramuscularly. Eighteen rats received both turpentine and cobalt in the amounts stated above. Only fourteen of the thirty-six rats receiving turpentine survived.

Methods—The wet digestion of the whole rat, the dry ashing of the wet digest, and the separation and measurement of the radioactive iron were carried out as described in Paper X (2). Hemoglobin determinations were made on tail blood at weekly intervals by the oxyhemoglobin method and with the Evelyn photoelectric colorimeter.

TABLE I

Effect of Turpentine, Cobalt, and Cobalt Plus Turpentine

The results are expressed in counts per minute.

Rat No.	Controls		Turpentine-treated		Cobalt-treated		Cobalt + turpentine-treated	
	Per rat	Per 100 gm.	Per rat	Per 100 gm.	Per rat	Per 100 gm.	Per rat	Per 100 gm.
1	972	954	832	650	1308	897	1020	784
2	1080	940	716	519	1144	817	1060	768
3	856	586	796	556	1220	813	908	745
4	1104	788	640	478	1104	725	1084	810
5	960	667	1052	756	988	667	1284	972
6	1272	889	988	667	1188	766	1432	1094
7	816	587	728	509	1480	1058	1240	885
8	976	646			988	772		
9	1056	714			784	586		
10					1192	832		
11					1168	880		
12					1236	812		
Mean.....	1010	752	822	591	1150	802	1146	865
Weight, gm.....	136 ± 17		139 ± 7		143 ± 9		132 ± 6	
Hb, gm. per 100 ml.....	16.4 ± 0.66		14.4 ± 2.67		20.3 ± 1.01		18.6 ± 1.76	

Results

The results are presented in Table I. The values for the body weight and final hemoglobin represent the averages for the groups; those for the total body radioactive iron in counts per minute are given in detail for each rat.

The rats injected with turpentine developed significant anemia in the 32 day period, whereas not only the cobalt-treated rats but also those receiving turpentine plus cobalt developed a significant degree of polycythemia in that time as judged by the hemoglobin values. Accompanying the decrease in hemoglobin production shown by the turpentine-

injected rats there was a decrease in iron absorption as demonstrated by the low total radioactive iron content of the body compared to the controls. The difference in counts per minute between the control and turpentine-injected groups is significant by Fisher's *t* test (3) ($t = 2.48$). This is true whether the counts are figured per rat or per 100 gm. of rat.

The rats given cobalt and those receiving cobalt plus turpentine showed a somewhat greater total body content of radioactive iron than the controls, but the differences are not significant. However, the difference between the turpentine and turpentine plus cobalt groups is highly significant ($t = 6.27$).

DISCUSSION

These experiments confirm our previous observation (1) that anemia does not develop following the production of turpentine abscesses if cobalt is injected. They are also consistent with the observation (4-6) that anemia associated with infection in human subjects can be influenced by the administration of cobalt. It is evident from the data, furthermore, that sterile abscesses, like bacterial abscesses and infections (2), are associated with a decrease in the absorption of iron from the gastrointestinal tract. The administration of cobalt to animals with sterile abscesses not only abolished the anemia-producing effects of turpentine and produced a polycythemia, but it also increased the absorption of iron from the gastrointestinal tract to within the normal range. In the cobalt-treated animals, most of the iron for the synthesis of the additional hemoglobin came from the gastrointestinal tract, even though the absorption of iron was not increased beyond normal in this group. This is in keeping with, and helps to explain, the observation that cobalt does not produce polycythemia when the diet is deficient in iron (7).

From this it must not be concluded that the principal action of cobalt is on the absorption of iron and that polycythemia is a result of this action. If this were true, the administration of iron parenterally should result in polycythemia, and such is not the case. It is more logical to presume that the action of cobalt is exerted in some other way and that, as a result of this, more iron is absorbed. Orten and Bucciero (8) have suggested that cobalt may produce polycythemia by binding sulfhydryl or perhaps other groups active in cellular respiration, thus leading to a simulated cellular anoxia and, in turn, to a compensatory polycythemia. If this is true, then, just as in hemorrhagic anemia, the rate of hemoglobin synthesis is accelerated, the need of the body for iron is increased, and the absorption of iron is enhanced.

Although cobalt completely counteracted the anemia-producing effects of turpentine and restored the amount of iron absorbed to normal, this

substance, in the presence of a turpentine abscess did not produce the same degree of polycythemia as in the animals treated with cobalt alone. This was true, even though the absorption of iron in the animals treated with cobalt and turpentine was approximately equal to that of the animals treated with cobalt alone. Thus cobalt must have produced a greater increase in tissue iron in animals injected with turpentine than in animals treated with cobalt alone.

SUMMARY

The influence of turpentine-induced abscesses and of cobalt administration on the absorption of iron has been investigated in rats by determining the total amount of radioactive iron retained in the body during the period of treatment.

Turpentine was found to decrease the absorption of iron, and this effect could be reversed by the administration of cobalt.

Cobalt administration did not significantly affect the absorption of iron in normal rats.

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THE ANEMIA OF INFECTION

XII. THE EFFECT OF TURPENTINE AND COLLOIDAL THORIUM DIOXIDE ON THE PLASMA IRON AND PLASMA COPPER OF DOGS*

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In an earlier report from this laboratory (1) the production of an acute hypoferremia in dogs, following the intramuscular injection of turpentine, was described. The hypoferremia so produced persisted for about 24 hours and then disappeared.

Since it is generally assumed that iron is withdrawn from the circulation by the cells of the reticulo-endothelial system, it seemed of interest to determine whether the administration of a "reticulo-endothelial blocking agent" such as colloidal thorium dioxide (2-9) would result in a hyperferremia and, if so, whether such a "blockade" might abolish the acute hypoferremia-producing effect of turpentine. At the same time it was proposed to observe the effects of the administration of thorium dioxide on the copper content of plasma and the iron-binding capacity of the serum.

Methods

Large, adult, mongrel dogs were used. Turpentine (rectified oil of turpentine, Rexall, U. S. P.) was given intramuscularly in the buttocks in a dose of 5 ml. Thorium dioxide (Thorotrast, Heyden Chemical Corporation, New York) was administered intravenously as a 25 per cent colloidal solution in amounts of 2 ml. per kilo of body weight per day. Determinations of plasma iron were made by the method of Barkan and Walker (10) with the Beckman spectrophotometer. Plasma copper and the iron-binding capacity of the serum were determined by the methods previously described by this laboratory (11, 12). The method of Schwartz, Sborov, and Watson (13) was used for the measurement of urinary and fecal urobilinogen. Specimens were obtained by 3 day collections. Serum bilirubin was determined by the Ducci and Watson (14) modification of the Malloy and Evelyn (15) method with the Evelyn photoelectric colorimeter.

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Results

Effect of Single Injection of Turpentine on Plasma Iron and Copper— The intramuscular injection of 5 ml. of turpentine is followed by rather characteristic changes in the plasma iron. A marked hypoferremia appears within 24 hours and reaches a maximum within 24 to 48 hours (Table I). Compared with the value just prior to the injection of turpentine, the decrease in the plasma iron during this period averaged 77 per cent (59 to 89). This hypoferremic phase is followed by a hyperferremic phase of brief duration which reaches a maximum on about the 8th day after injection. By about the 12th day following injection of turpentine the

TABLE I
Effect of the Intramuscular Injection of 5 Ml. of Turpentine on Plasma Iron

Dog No.	Plasma iron			Time of maximum decrease
	Initial	Lowest subsequent value	Decrease	
	<i>γ per cent</i>	<i>γ per cent</i>	<i>per cent</i>	
1	160	40	75	24
2	168	43	74	24
3	210	36	83	48
4	180	38	79	48
5	192	45	76	24
6	229	35	85	24
7	200	22	89	48
8	142	40	72	24
9	150	40	73	24
10	110	45	59	48
Mean.....			77	

plasma iron returns to normal (Fig. 1). The plasma copper level is essentially unaffected by the injection of turpentine (Figs. 1 and 3, 4).

Effect of Single Injection of Colloidal Thorium Dioxide on Plasma Iron— A single intravenous injection (2 ml. per kilo of body weight) of colloidal thorium dioxide was given to thirteen dogs (Table II). In ten of the dogs (77 per cent) this was followed in 24 hours by a moderate hypoferremia of 36 to 79 per cent. In three animals (23 per cent) there was an increase in the plasma iron. The average per cent change in plasma iron for the entire group was a decrease of 29 per cent.

*Effect of Three Consecutive Daily Intravenous Injections of Colloidal Thorium Dioxide on Plasma Iron and Copper—*The administration of colloidal thorium dioxide to nine dogs for 3 consecutive days resulted in an increase in the plasma iron (Table III). This increase generally began on the 1st

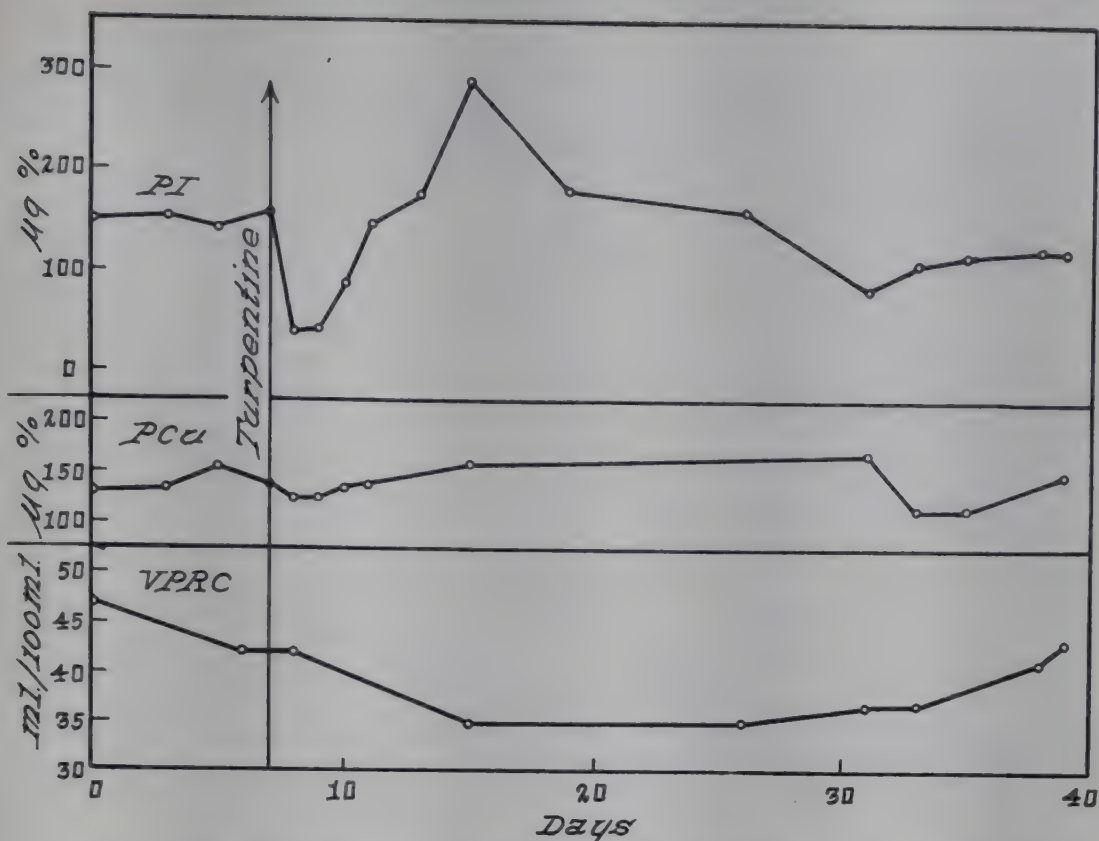


FIG. 1. Effects produced by the intramuscular injection of 5 ml. of turpentine on the plasma iron (PI), plasma copper (PCu), and the volume of packed red cells (VPRC) of a dog.

TABLE II

Effect of Single Intravenous Injection (2 Ml. per Kilo) of Colloidal Thorium Dioxide on Plasma Iron

Dog No.	Plasma iron		
	Initial	Final	Change*
	γ per cent	γ per cent	per cent
1	245	52	-79
2	265	25	-91
3	255	100	-61
4	140	88	-37
5	150	96	-36
6	130	277	+113
7	285	78	-73
8	240	145	-40
9	60	90	+50
10	83	103	+24
11	205	95	-54
12	220	125	-43
13	175	82	-53
Mean.....			-29

* 24 hours after injection.

TABLE III

Effect of Three Consecutive Daily Intravenous Injections (2 Ml. per Kilo) of Colloidal Thorium Dioxide on Plasma Iron

Dog No.	Plasma iron			Time of maximum increase
	Initial	Highest subsequent value	Increase	
	γ per cent	γ per cent	per cent	days
1	175	210	20	3
2	130	800	515	5
3	210	780	271	5
4	140	215	54	7
5	240	625	161	4
6	60	137	128	6
7	82	822	904	7
8	150	877	485	3
9	71	1122	1481	4
Mean.....			446	

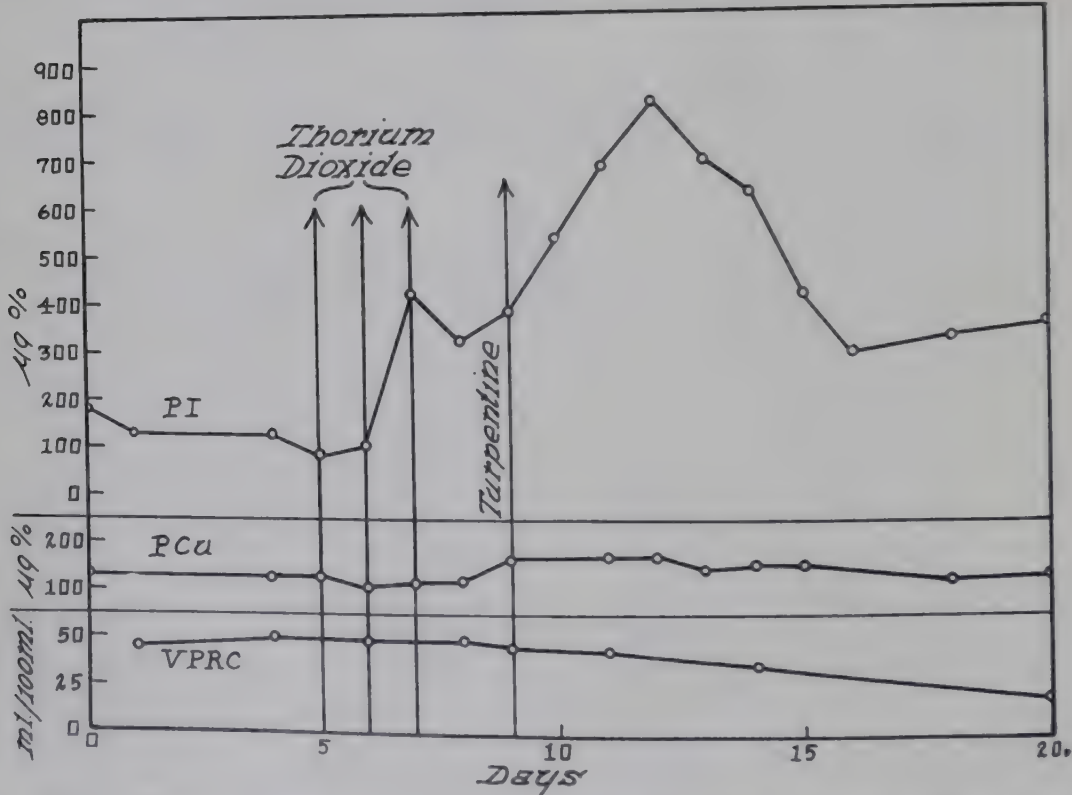


FIG. 2. Effects of three intravenous injections of colloidal thorium dioxide (2 ml. per kilo of body weight) on the plasma iron (PI), plasma copper (PCu), and volume of packed red cells (VPRC) of a dog. Note the failure of turpentine (5 ml. intramuscularly) to produce hypoferrremia when administered after the thorium.

day following the last injection and reached a maximum on the 3rd to 7th day (Fig. 2). The per cent increase in plasma iron varied considerably (20 to 1481 per cent) from dog to dog, but averaged 446 per cent. The hyperferremia lasted about 6 days, but varied in duration from 4 to 12 days. No change in the plasma copper was observed (Fig. 3, B).

No untoward effects were observed in any of the dogs.

Effect of Intramuscular Injection of 5 Ml. of Turpentine in Dogs Previously Given Three Daily Intravenous Injections of Colloidal Thorium Dioxide—Five dogs were given an intramuscular injection of turpentine 2 to 5 days after the last of three daily intravenous injections of thorium (Table IV, Figs. 2 and 3, C). In four of the dogs there was a slight but in-

TABLE IV

Effect of Intramuscular Injection of 5 Ml. of Turpentine in Dogs Previously Given Colloidal Thorium Dioxide

Dog No.	Plasma iron			Time of maximum decrease*
	Initial	Lowest subsequent value	Decrease	
	γ per cent	γ per cent	per cent	hrs.
1	350	260	26	24
2	130	105	19	48
3	380	540	0	
4	145	130	10	24
5	153	103	33	24
Mean.....			18	

* After turpentine injection.

significant decrease in the plasma iron. In no instance did the plasma iron decrease to below 100 γ per cent. In one dog the turpentine was administered while the plasma iron was increasing (Fig. 2). In this animal there was no decrease in the plasma iron. In no instance was the decrease comparable to that following the injection of turpentine alone (Table I, Fig. 1). There was no significant change in the plasma copper when turpentine was given following thorium administration (Figs. 2 and 3, C).

Effect of Turpentine, Colloidal Thorium Dioxide, and Turpentine Following Colloidal Thorium Dioxide on Iron-Binding Capacity of Serum, Serum Bilirubin, and Urobilinogen Excretion—The administration of turpentine, as reported previously (8), results in a significant decrease in the total iron-binding capacity of the serum (Fig. 4, A). Repeated injections of

thorium were followed by a considerable increase in the total iron-binding capacity (Fig. 4, B). The administration of turpentine following thorium failed to decrease this component significantly (Fig. 4, C).

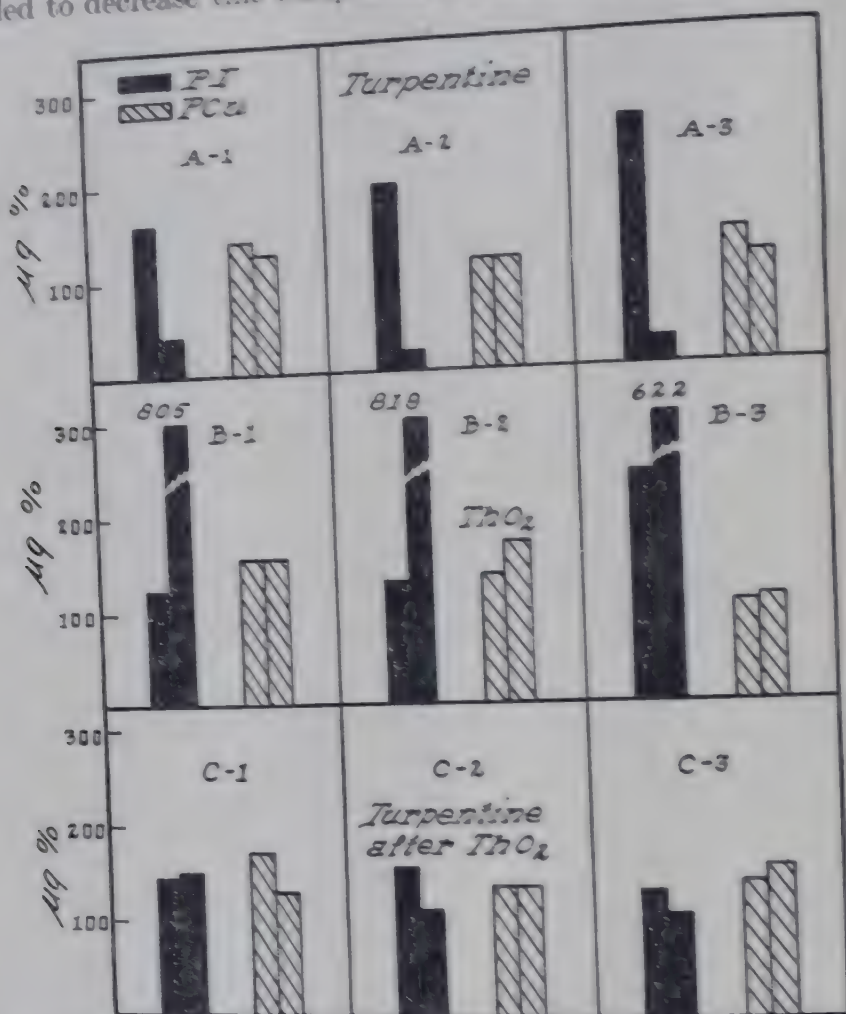


FIG. 3. Changes in plasma iron (solid bars) and plasma copper (hatched bars). A, 5 ml. of turpentine injected intramuscularly into three dogs (A-1, A-2, A-3). B, three intravenous injections (2 ml. per kilo of body weight) of colloidal thorium dioxide daily. C, turpentine given after three injections of colloidal thorium.

3 day urine and stool collections, for the purpose of measuring urobilinogen, were made on three dogs for a period of 9 days. One dog was then given an injection of turpentine, the second dog received three injections of thorium, and the third dog was given three injections of thorium followed by turpentine. For the control periods the average urinary urobilinogen excretion was 0.2 mg. per 24 hours and the stool urobilinogen averaged 23 mg. per 24 hours. No significant increase or decrease in the urobilinogen content of the urine or feces was noted following the admin

istration of turpentine or thorium, or thorium plus turpentine. The serum bilirubin remained below 0.4 mg. per cent in all three animals.

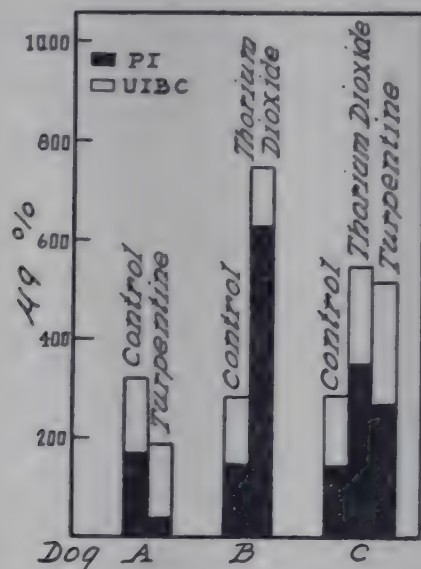


FIG. 4. Changes in plasma iron (solid), unbound iron-binding capacity, and total iron-binding capacity of the serum ($PI + UIBC$) in a dog before and after the injection of turpentine (A) and of colloidal thorium (B), and in another dog (C) given turpentine after colloidal thorium.

DISCUSSION

The experimental data indicate that the repeated injection of colloidal thorium dioxide into dogs resulted in a marked hyperferremia and that under these circumstances the hypoferremia-producing effects of turpentine were abolished. Since it is known that injected colloidal thorium is to be found largely in the macrophagic tissue (16), and that the injection of colloidal substances results in at least a temporary depression of macrophagic function (5, 17), it seems reasonable to conclude from the above experiments that the administration of repeated doses of colloidal thorium resulted in a temporary functional alteration in the reticulo-endothelial system, at least in so far as iron metabolism is concerned. If this is true, then it is possible that the injection of turpentine (without previous Thorotrast administration) in some way enhances or stimulates the functional state of the reticulo-endothelial system so that the iron is rapidly removed from the serum and hypoferremia results.

It should be stated, however, that we do not regard the data presented as giving conclusive proof that the hyperferremic effect of the colloidal thorium is due entirely or in part to a functional "blockage" of the reticulo-endothelial system. Objective quantitative methods for the measurement of the functional state of this system are not available. Whether

the injection of colloids, particles, or vital dyes actually results in a significant depression of histiocyte function has been and still remains a controversial subject (5, 17). It is quite possible that the hyperferremic effect of thorium may be through another mechanism.

Vannotti and Imholz (6), measuring total non-hemoglobin iron (serum iron plus "easily split off" iron), observed a marked fall after a single injection of thorium as well as after several injections at short intervals. After long continued injections or after repeated injections in splenectomized animals they observed a marked increase in non-hemoglobin iron. Their experiments are difficult to compare with ours since they used rabbits rather than dogs, measured total non-hemoglobin iron rather than serum iron, and failed to state clearly the quantity of thorium injected. They interpreted the hyperferremia they observed as being the result of increased hemolysis due to increased red cell phagocytosis by the reticulo-endothelial system. Studies on the excretion of urobilinogen were not reported. The results of our measurements on urobilinogen excretion do not support the concept that increased blood destruction is the cause of the hyperferremia. A more likely explanation would seem to be that the rate of uptake of iron from the serum by the reticulo-endothelial system was reduced.

Recent work by Gordon and Katsh (16) suggests that the adrenal cortex exerts a regulatory influence upon the structure and function of the macrophagic cells. Experiments designed to determine whether alteration of the functional state of the reticulo-endothelial system by this means has any effect on the plasma iron are now in progress. Earlier studies in rats (18) failed to reveal any relationship of the adrenal cortex to the changes in plasma iron.

Plasma copper levels were essentially unaffected by either turpentine or colloidal thorium injection. This would seem to indicate that the metabolic pathway followed by copper is quite different from that of iron.

SUMMARY

1. The intramuscular injection of 5 ml. of turpentine into each of ten dogs resulted in an average decrease in plasma iron of 77 per cent within 24 to 48 hours.

2. The intravenous injection of a single dose of colloidal thorium dioxide (2 ml. per kilo of body weight) into each of thirteen dogs resulted in an average decrease in plasma iron of 29 per cent in 24 hours.

3. The administration of three consecutive daily intravenous injections of colloidal thorium dioxide into each of nine dogs resulted in an average increase in the plasma iron of 446 per cent within 3 to 7 days.

4. The intramuscular injection of 5 ml. of turpentine into each of five

dogs previously given three consecutive daily intravenous injections of colloidal thorium dioxide resulted in an average decrease in plasma iron of only 18 per cent.

5. These studies suggest the possibility that the hypoferremia which follows the intramuscular injection of turpentine is the result of increased activity of the reticulo-endothelial system.

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THE USE OF ISOTOPIC NITROGEN IN A STUDY OF THE CONVERSION OF 3-HYDROXYANTHRANILIC ACID TO NICOTINIC ACID IN *NEUROSPORA**

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Recent studies (1) with mutant strains of *Neurospora* indicated that 3-hydroxyanthranilic acid (3-hydroxy-2-aminobenzoic acid) is an intermediate in the metabolic conversion of tryptophan to nicotinic acid. Evidence for this conversion in mammals is found in the report (2) that 3-hydroxyanthranilic acid can maintain the growth of rats on a nicotinic acid-deficient diet and in the work of Albert, Scheer, and Deuel (3), who found an increased excretion of *N*-methylnicotinamide by rats fed this substance. The isotopic experiments of Heidelberger, Abraham, and Lepkovsky (4) demonstrated that the carboxyl carbon of 3-hydroxyanthranilic acid is the precursor of the carboxyl carbon atom of nicotinic acid.

To study the mechanism of the conversion of the benzene ring into a pyridine derivative, a mutant strain of *Neurospora crassa* C-86, requiring 3-hydroxyanthranilic acid or a precursor for growth, was grown in a medium which contained *non-labeled* synthetic (5) 3-hydroxyanthranilic acid and N^{15} ammonium chloride as the only sources of nitrogen. Thus the absence of N^{15} in excess in nicotinic acid isolated from the mold would indicate that the amino group of 3-hydroxyanthranilic acid is the precursor of the pyridine nitrogen in nicotinic acid.

EXPERIMENTAL

The experiment was performed in duplicate.

A 5 liter culture of *Neurospora* mutant strain C-86 was grown 4 days at 25° under forced aeration. The culture medium was made up of the following components: 10 gm. of $NaH_2PO_4 \cdot H_2O$, 25 gm. of potassium tartrate, 2.5 gm. of $MgSO_4$, 0.5 gm. of $CaCl_2$, 0.5 gm. of $NaCl$, 25 gm. of

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biotin, 5 ml. of a solution of trace elements, 50 gm. of sucrose, 1.3 gm. of NH_4Cl (N^{15} -58 atom per cent excess obtained from the Eastman-Kodak Company as nitrate and converted to the chloride), 6.6 mg. of 3-hydroxyanthranilic acid (*non-tagged*), and 5 liters of H_2O . The pH of the medium was made 5.0 with concentrated hydrochloric acid. At the end of the growth period, the mold was filtered through cheese-cloth and continuously extracted with acetone for 16 hours.

Isolation of Nicotinic Acid—The acetone extract of the mycelium was evaporated to 1.5 ml. and shaken with 4 ml. of absolute ethanol. The alcohol precipitated a considerable amount of material which was filtered off. The acetone extract contained 900 γ (Experiment 1) of niacin activity before the alcohol precipitation and 700 γ after precipitation.¹ Niacin activity was determined in triplicate by bioassay with *Lactobacillus arabinosus* strain 17-5 and is accurate to ± 4 per cent. The filtrate from the alcohol precipitation of the acetone extract was dried and heated for 50 minutes at 95° in 10 ml. of an aqueous $\text{Ba}(\text{OH})_2$ solution (50 per cent saturated at 100°). The baryta solution was diluted with water, titrated with sulfuric acid to pH 3, and filtered. The filtrate was made pH 5.2 with NH_4OH , concentrated to a volume of about 0.5 ml., and placed on a 35 cm. paper chromatogram (Whatman No. 1). The chromatogram, developed 16 hours in a solution of *n*-butanol saturated with 0.2 N ammonium hydroxide, gave an ascending solvent front of 25.5 cm. above the point of sample deposition. A 2 mm. vertical strip cut from the center of the chromatogram was further cut into 1 cm. segments and numbered consecutively, starting at the point of sample deposition. Each 2×10 mm. strip was assayed on 5 ml. cultures of *Lactobacillus arabinosus*. The only detectable activity for niacin was found on Strips 4, 5, and 6. The corresponding strips from the total chromatogram yielded 600 γ (Experiment 1) and 190 γ (Experiment 2) of nicotinic acid when eluted with water. To these samples of isolated nicotinic acid 40 mg. of stock nicotinic acid (unlabeled) were added. This material was recrystallized twice from absolute ethyl alcohol and then sublimed at 170 – 180° .

Samples of the mycelium and nicotinic acid were converted to nitrogen by the method described by Rittenberg, Keston, Rosebury, and Schoenheimer (6) and analyzed for N^{15} content with a mass spectrometer.²

Results

The results of the experiments are summarized in Table I. All mass spectrometer analyses were carried out in duplicate with a precision of ± 1 per cent.

¹ No preliminary assays on Experiment 2 prior to isolation by chromatography.

² We are indebted to Dr. Sidney Soloway of the Public Health Research Institute of the City of New York for the mass spectrometer analyses.

The data obtained would indicate that 54 per cent (the average value of Experiments 1, b and 2, c) of the nitrogen in nicotinic acid is derived from the amine nitrogen of 3-hydroxyanthranilic acid. The closeness of this value to 50 per cent may indicate that the transformation of 3-hydroxyanthranilic acid to nicotinic acid is not a simple one and that a yet unidentified symmetrical compound may be an intermediate. It might be hypothesized that a second amino group is attached to 3-hydroxyanthranilic acid in the 4 or 6 position before the conversion of the benzene to the pyridine ring occurs. Such a mechanism would be considerably different from that recently proposed by Bonner and Yanofsky (7) to account for the accumulation of quinolinic acid by a *Neurospora* mutant. It

TABLE I
N¹⁵ Analyses

Experiment No.	Sample	Mass spectrometer analysis, atom per cent N ¹⁵ excess	Dilution factor in purification	Corrected, atom per cent N ¹⁵ excess	Per cent of nitrogen activity from N ¹⁵ H ₄ Cl	Per cent of pyridine nitrogen from 3-hydroxyanthranilic acid
1, a	Mycelium, 5.5 gm. dry weight	58.3	1	58.3	100	0
1, b	Isolated nicotinic acid (m.p. 234–237°)	0.421	40.6/0.6	28.5	49	51
2, a	Mycelium 4.8 gm. dry weight	58.1	1	58.1	100	0
2, b	Isolated nicotinic acid (m.p. 231–234°), unpurified	0.112	40.2/0.19	23.7	41	59
2, c	Isolated nicotinic acid (m.p. 234–236°), purified	0.117	40.2/0.19	24.8	43	57

is possible that the mutant used in the present work has the capacity for synthesis of part of the niacin required, since recent evidence (8) indicates that this mutant does have a partial genetic block. It seems unlikely, however, that 50 per cent of the niacin isolated can be derived in this fashion because of the tryptophan cycle (8) and because of the fact that strain C-86 requires as much niacin as other mutants that evidently do not have partial synthetic capacities.

The appearance of unlabeled nitrogen in the nicotinic acid is further evidence for the conversion of 3-hydroxyanthranilic acid to nicotinic acid.

SUMMARY

1. Further support for the conversion of 3-hydroxyanthranilic acid to nicotinic acid in *Neurospora* is demonstrated by a tracer experiment with N¹⁵ ammonium chloride.

2. The experimental findings suggest a symmetrical diamino compound as an intermediate in the conversion since nearly half of the nicotinic acid nitrogen is derived from $N^{15}H_3$ in the culture medium.

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ISOLATION OF Δ^{16} -ANDROSTEN-3(α)-OL FROM THE URINE OF WOMEN WITH ADRENAL CORTICAL TUMORS

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In a recent paper (1) brief mention was made of the isolation of an unsaturated steroid alcohol, which was assigned the formula $C_{21}H_{34}O$. This formula was based on analysis of the substance and its acetate, both of which were sublimed in a high vacuum at temperatures between 90–110° in preparation for the analysis. Although comparison of the analytic values for the alcohol with those calculated for $C_{21}H_{34}O$ left something to be desired, analysis of the acetate supported the conclusion that the alcohol was a C_{21} compound with a single oxygen atom and probably one double bond. However, infra-red analysis has shown that the substance is Δ^{16} -androst-3(α)-ol. Its physical properties also agree with those given by Prelog, Ruzicka, and Wieland (2) for Δ^{16} -androst-3(α)-ol. The elementary analyses were not repeated, since the infra-red analysis was considered to be definitive.

In the first case in which this substance was encountered each 24 hour specimen of urine was extracted with butyl alcohol as soon as it was received in the laboratory. The combined extracts were redistributed between 0.1 N sodium hydroxide and butyl alcohol several times and the conjugates soluble in butyl alcohol were subjected to enzymic hydrolysis with rat liver powder (glucuronidase) (3). The free steroids were separated into ketonic and non-ketonic fractions with the aid of Girard's Reagent T and the non-ketonic fraction was chromatographed on a column of magnesium silicate (4). Δ^{16} -Androst-3(α)-ol was eluted with a 1:1 mixture of benzene and petroleum ether. None of the procedures used would be expected to cause loss of water from a compound with a 16-hydroxyl or 17-hydroxyl group to produce the Δ^{16} -unsaturation. The most drastic conditions to which the extract was subjected would appear to be the refluxing with alcohol, acetic acid, and Girard's Reagent. It is most likely that Δ^{16} -androst-3(α)-ol was present in the urine conjugated with glucuronic acid.

In the second case Δ^{16} -androst-3(α)-ol was isolated from the extract obtained after boiling the urine with hydrochloric acid (1). It is conceivable that Δ^{16} -unsaturation may have occurred during the boiling with acid or later during chromatographic separation on alumina.

The substance isolated from urine in the first instance melted at 145°

after repeated recrystallizations and sublimation; $[\alpha]_D^{26} = +20.9^\circ \pm 1.3^\circ$ (*c* 0.693 in alcohol); $[\alpha]_D^{28} = +14.5^\circ \pm 1^\circ$ (*c* 1.034 in chloroform). A melting point of $140\text{--}141^\circ$ was given for the material isolated in the second case (1), but it should have been $142\text{--}143^\circ$. Prelog and associates (2) recorded a melting point of $143.5\text{--}144^\circ$ and $[\alpha]_D^{16} = +13.9^\circ \pm 2^\circ$ (*c* 0.936 in chloroform) for synthetic Δ^{16} -androsten-3(α)-ol. The urinary substance formed an acetate which melted at $132\text{--}133^\circ$ after sublimation; $[\alpha]_D^{26} = +20.5^\circ \pm 1.7^\circ$ (*c* 0.487 in alcohol). The benzoate melted at 142° ; $[\alpha]_D^{26} = 0.0^\circ \pm 2.2^\circ$ (*c* 0.450 in alcohol). These derivatives and the corresponding ones of androstan-3(α)-ol have not been described previously.

The urinary substance was reduced with platinum and hydrogen to give androstan-3(α)-ol melting at $145\text{--}146^\circ$ (Prelog and associates (2) observed a melting point of $145\text{--}146^\circ$). The acetate and benzoate melted at $128\text{--}129^\circ$ and $148\text{--}150^\circ$, respectively. Oxidation with chromic acid gave androstan-3-one, which melted at $97\text{--}98^\circ$ (Prelog and associates (5) recorded a melting point of $104.5\text{--}105.5^\circ$, and Heard and McKay (6), $102\text{--}102.5^\circ$) and formed a yellow dinitrophenylhydrazone (m.p. $235\text{--}236^\circ$).

Oxidation of the urinary substance with Oppenauer's reagents gave Δ^{16} -androsten-3-one, which melted at $141\text{--}142^\circ$ (Prelog and associates (2), $140\text{--}141^\circ$) and formed a yellow 2,4-dinitrophenylhydrazone melting at $167\text{--}168^\circ$ and an oxime melting at $158\text{--}159^\circ$ (Prelog and associates (5), $163\text{--}165^\circ$).

Although almost all of the properties of the urinary substance and its derivatives agree with those found by Prelog and associates (2, 5) for Δ^{16} -androsten-3(α)-ol and its derivatives, the decisive factor in the identification is the infra-red spectrogram.¹ The identity of the spectrograms of Δ^{16} -androsten-3(α)-ol and of the urinary substance leaves no doubt as to the identity of these substances (Fig. 1).

Δ^{16} -Androsten-3(α)-ol was isolated by Prelog and Ruzicka (7) from extracts of swine testes. The methods used were such as to leave some doubt whether the compound was present in the testes as such or whether it might be an artifact. Brooksbank and Haslewood (8) obtained this compound from normal male urine in the form of the glucuronide, from

¹ We are indebted to Dr. Konrad Dobriner, Sloan-Kettering Institute, for the infra-red spectrograms and the identification of Δ^{16} -androsten-3(α)-ol. The authentic specimen of Δ^{16} -androsten-3(α)-ol was obtained by Dr. Dobriner from Dr. Prelog, who had isolated it from hog testes. Dr. Dobriner kindly furnished us with a small sample of Prelog's material and of the crude acetate which had been used for examination of the infra-red spectrum. The acetate was recrystallized once from methanol. It melted at $128\text{--}129^\circ$ and the melting point was not depressed by admixture of the acetate of the urinary material (this sample melted at $129\text{--}130^\circ$). The melting point of the material from hog testes also was not depressed by admixture of the urinary substance.

which it was liberated by incubation with *Staphylococcus albus*. There was no reason to think that the bacteria had any effect other than that of hydrolysis of the glucuronide. In our first case there is reason to believe that the Δ^{16} -androst-3(α)-ol isolated from the urine was a product

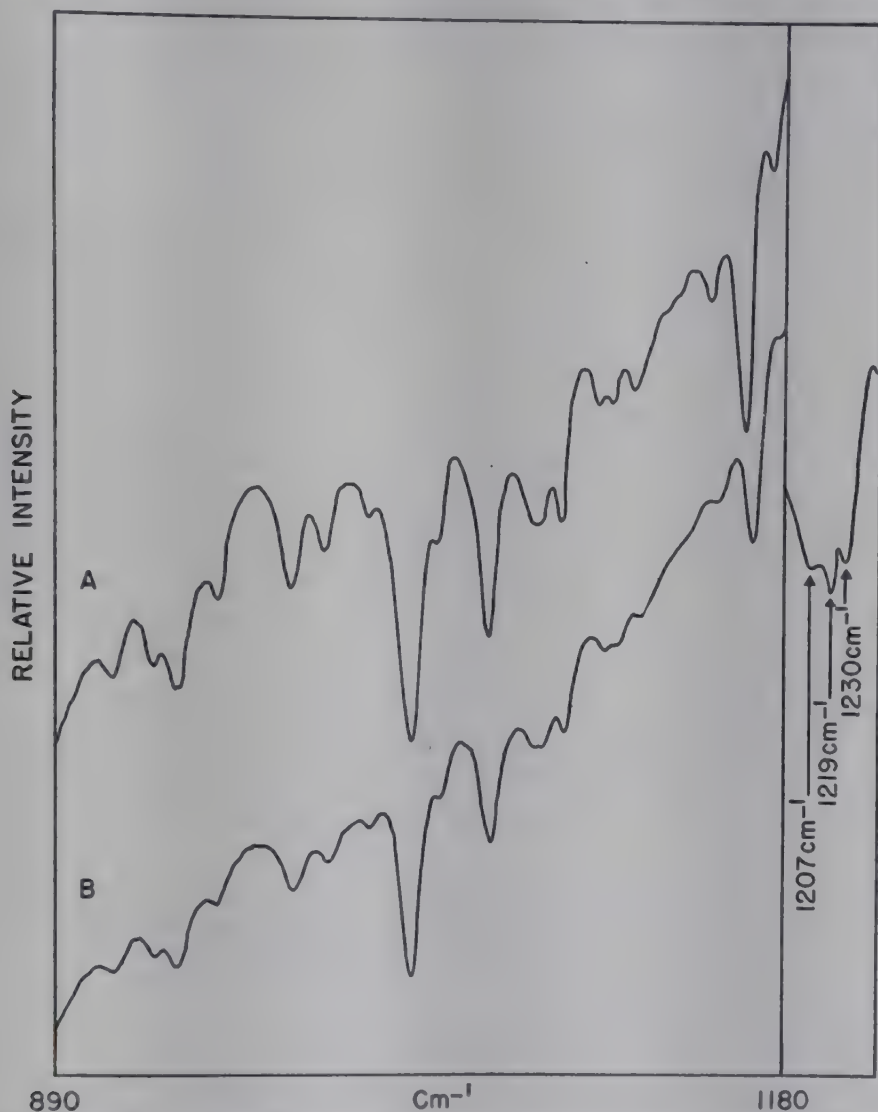


FIG. 1. Curve A, infra-red absorption spectrum of Δ^{16} -androst-3(α)-ol isolated by Prelog from swine testes. Curve B, infra-red absorption spectrum of urinary substance.

of the adrenal cortical neoplasm or a product of the metabolism of some precursor produced by the neoplasm. This unsaturated steroid appears to furnish another link between the testis and the adrenal cortex.

EXPERIMENTAL

Melting Points—All melting points were determined with a Fisher-Johns apparatus and are recorded as read.

Collection and Extraction of Urine—Most of the procedures used in collection and processing of the urine have been described (3). In the first case, however, an attempt was made to isolate steroid conjugates. Each 24 hour specimen of urine was extracted four times with 200 ml. of butyl alcohol per liter. Isolation of steroids from the residual urine was described by Mason and Kepler (3), Case 5. The pooled butyl alcohol extracts obtained during 37 days were evaporated to dryness in a vacuum. The residue was dissolved in 500 ml. of water and the solution was extracted with chloroform to remove any free steroids. 10 ml. of 5.0 N sodium hydroxide were then added and the solution was extracted exhaustively with butyl alcohol. The combined extracts were washed twice with 25 ml. of water and distilled to dryness. The distribution between 0.1 N sodium hydroxide and butyl alcohol was repeated twice more with addition of 10 gm. of sodium chloride to facilitate separation of phases. The final butyl alcohol extract was concentrated in a vacuum until crystalline material began to separate. This material was identified as a mixture of sodium chloride and sodium dehydroisoandrosterone sulfate. After removal of several crops of crystals the butyl alcohol was removed, the residue was dissolved in water, and the water was partially distilled in a vacuum to help remove any remaining butyl alcohol. The aqueous solution of steroid conjugates was divided into several portions and incubated with rat liver powder as previously described (3). The aqueous residues remaining after incubation and extraction were further extracted with butyl alcohol and this extract was again incubated with rat liver powder. The neutral fraction was worked up in the usual manner (3).

Isolation of Δ^{16} -Androsten-3(α)-ol—In the first case the non-ketonic fraction (3.43 gm.) was dissolved in 60 ml. of benzene, and 60 ml. of petroleum ether were added. This solution was placed on a column of 101 gm. of magnesium silicate and infusorial earth (3:5), 4 cm. by 27 cm. The column was then washed with a 1:1 mixture of benzene and petroleum ether. The first 300 ml. removed 38 mg. of amorphous material. The next 700 ml. removed 646 mg. of crystalline material. Recrystallization from acetone gave 481 mg. which melted at 144° . A second crystallization from acetone did not change the melting point; $[\alpha]_D^{28} = +20.9^\circ \pm 1.3^\circ$ (c 0.693 in 95 per cent alcohol); $[\alpha]_D^{28} = +14.5^\circ \pm 1^\circ$ (c 1.034 in chloroform). Since the twice recrystallized product gave a slightly turbid solution in alcohol, a portion was sublimed in a high vacuum at $90-95^\circ$ bath temperature for an analytic sample. The melting point was then 145° .

$C_{19}H_{30}O$. Calculated, C 83.15, H 11.02; found, C 83.96, H 11.00
 $C_{21}H_{34}O$. " " 83.38, " 11.33

In the second case, the alcoholic non-ketonic fraction was chromatographed on a column of alumina (Fisher's alumina for chromatography). Benzene eluted a crystalline fraction which proved to be identical with that just described.

Δ^{16} -Androsten-3(α)-ol Acetate—This derivative was prepared by heating 25 mg. of the alcohol with 10 drops of pyridine and 5 drops of acetic anhydride at 90° for 30 minutes. After cooling, water and dilute hydrochloric acid were added and the precipitate was collected on a filter, washed with water, dried, crystallized from methanol, and sublimed in a high vacuum at 100–110°. The acetate melted at 132–133° and $[\alpha]_D^{26} = +20.5^\circ \pm 1.7^\circ$ (*c* 0.487 in 95 per cent alcohol).

$C_{21}H_{32}O_2$. Calculated, C 79.70, H 10.67; found, C 80.30, H 10.86
 $C_{23}H_{36}O_2$. " " 80.18, " 10.53

Δ^{16} -Androsten-3(α)-ol Benzoate—The benzoate was prepared in the same manner as the acetate with benzoyl chloride and pyridine. After two crystallizations from methanol it melted at 142°; $[\alpha]_D^{26} = 0.0^\circ \pm 2.2^\circ$ (*c* 0.450 in 95 per cent alcohol).

Catalytic Reduction. Androstan-3(α)-ol—When 50.9 mg. of the urinary substance dissolved in 10 ml. of absolute alcohol were shaken in an atmosphere of hydrogen with platinum catalyst (40 mg. of platinum oxide reduced just prior to addition of the steroid), uptake of hydrogen was rapid and ceased within 20 minutes. The hydrogen absorbed was 3.72 ml.; calculated for one double bond, 3.77 ml. The catalyst and solvent were removed and the residue was crystallized twice from dilute acetone. The androstan-3(α)-ol melted at 145–146° and did not depress the melting point of the starting material. That the unsaturated alcohol had been reduced was shown by the work which follows.

Androstan-3(α)-ol Acetate and Benzoate—These derivatives were prepared as before. The acetate melted at 128–129° and a mixture with the acetate of Δ^{16} -androsten-3(α)-ol melted at 124–125°. The benzoate melted at 148–150°. A mixture with Δ^{16} -androsten-3(α)-ol benzoate (m.p. 139–140°) melted at 140–142°.

Androstan-3-one—Oxidation of 30 mg. of androstan-3(α)-ol in 0.5 ml. of glacial acetic acid with 2.1 equivalents of chromium trioxide in 0.25 ml. of 90 per cent acetic acid for 12 hours at room temperature gave 30 mg. of neutral product. After crystallization from methanol it melted at 97–98°. It readily formed a yellow dinitrophenylhydrazone which melted at 235–236°.

Δ^{16} -Androsten-3-one—The urinary substance (40 mg.) was refluxed for 12 hours under anhydrous conditions with 120 mg. of aluminum tertiary butoxide, 3 ml. of cyclohexanone, and 5 ml. of benzene. The crude prod-

uct was chromatographed on a column of 2.5 gm. of alumina. Petroleum ether eluted 22 mg. of material, which was recrystallized from methanol. It melted at 141–142° and a mixture with the starting material (m.p. 142–143°) melted at 107–110°. It formed a yellow dinitrophenylhydrazone which melted at 167–168° and an oxime which melted at 158–159°.

SUMMARY

Δ^{16} -Androsten-3(α)-ol has been isolated from the urine of two women with adrenal cortical tumors. In one case the isolation procedures were such as to make it unlikely that the Δ^{16} -unsaturation was produced by any of those procedures. Δ^{16} -Androsten-3(α)-ol was oxidized to Δ^{16} -androsten-3-one and reduced to androstan-3(α)-ol, which in turn was converted to androstan-3-one. The acetates and benzoates of the two alcohols and the 2,4-dinitrophenylhydrazones of the ketones were prepared.

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THE AMINO ACID-SUGAR REACTION*

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In studies of methods for the determination of the nutritive value of parenteral protein hydrolysate solutions containing dextrose, it was observed that such preparations invariably had a lower nutritive value than was expected. In controlled experiments in which protein hydrolysates of known biological value were dried in the presence of glucose, browning and loss of nutritive value occurred even under mild conditions (1). It was demonstrated further by experiments with animal feeding that impaired nutritive value was restored by supplementation with the pure amino acids. Microbiological assay of the amino acid content of the impaired preparations showed reduced amino acid values, but the reduction was not as great as indicated by the results with animals. On the basis of these studies, we suggested the formation of an amino acid-glucose complex that is relatively less available to the rat than to the microorganism. The loss of nutritive value was accompanied by the occurrence of browning. The browning reaction has been studied in many laboratories by various techniques, and recently fluorescence has been described as a guide in measuring the course of the reaction (2-4). Dutton and Edwards have concluded that the colored compounds and the fluorescent compounds are identical (4). Barnes and Kaufman (5) confirmed this to the extent of showing that the color and fluorescence curves are parallel. They noted, however, that, in the presence of bisulfite used as an inhibitor of color formation, the ratio of fluorescence to color was about 5 times that of a sample without bisulfite.

It is our purpose here to report further studies on the amino acid-glucose reaction.

EXPERIMENTAL

Solutions of the ten amino acids usually listed as essential (6), and in addition cystine and tyrosine, were subjected to different conditions with

* Presented in part before the Division of Biological Chemistry of the American Chemical Society, Atlantic City, September 22, 1949.

† The material reported here is part of a thesis submitted by Leo Friedman in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry, April, 1949, in the Graduate School of Georgetown University, Washington, D. C.

and without glucose. Solutions of 0.05 M amino acids and 0.25 M glucose, in acetate buffer, pH 5.2, were heated at 15 pounds pressure (121°) for 30, 60, and 120 minutes. (Enough HCl was added to the cystine and tyrosine solutions to prevent precipitation, resulting in solutions of pH 2.1 and 2.5 respectively.) The heated solutions were diluted 1:20 and the following

TABLE I

Results of Measurements of Amino Acids with and without Glucose
Amino acids, 0.05 M, and glucose, 0.25 M, autoclaved at 15 pounds, 121°.

	Per cent change of amino acid from control without glucose			Optical density at 385 m μ				Fluorescence, per cent quinine*			
	30 min.	60 min.	120 min.	120 min.†	30 min.	60 min.	120 min.	120 min.†	30 min.	60 min.	120 min.
L-Tyrosine.....	-6	2	0	0.013	0.022	0.032	0.071	5	5	6	15
DL-Methionine.....	-1	0	0	0.018	0.097	0.276	1.40	8	69	124	70
L-Leucine.....	0	-6	-7	0.013	0.086	0.222	0.638	5	66	132	120
DL-Valine.....	-6	-7	-9	0.009	0.081	0.222	0.553	4	56	108	97
DL-Isoleucine.....	-8	-7	-11	0.018	0.071	0.200	0.602	5	61	115	105
L-Cystine.....	-2	-3	-14	0.041	0.076	0.149	0.456	10	24	53	48
DL-Threonine.....	0	+5	-22	0.009	0.076	0.194	0.602	7	71	150	154
L-Lysine.....	-11	-17	-25	0.009	0.252	0.770	1.82	8	193	143	31
DL-Phenylalanine.....	-2	-13	-27	0.022	0.161	0.456	1.15	8	160	218	108
L-Tryptophan.....	-10	-19	-37	0.027	0.638	1.460	2.30	11	593	460	251
L-Arginine.....	-10	-14	-44	0.022	0.066	0.143	0.409	10	88	121	160
L-Histidine.....	-16	-25	-44	0.009	0.260	0.770	1.700	10	268	340	160
Mixtures‡.....							0.328	40	81	175	352
Glucose (autoclaved 3 hrs.).....							0.097				22

* Fluorescence was measured with a Coleman electronic fluorophotometer (filter Nos. B-2 and PC-1). The exciting light source was a mercury vapor lamp. Fluorescence was compared in each case with a quinine sulfate solution containing 0.0005 mg. per ml. in 0.1 N H₂SO₄.

† Amino acid control (no glucose).

‡ Equal volumes of all the amino acid solutions mixed together, then treated in the same way as the individual amino acids.

measurements made directly on each: (1) microbiological assay of the amino acid content; (2) measurement of the ultraviolet absorption from 240 to 400 m μ in the Beckman DU spectrophotometer at 5 m μ intervals; (3) comparison of color intensity at 385 m μ ; and (4) determination of the degree of fluorescence by comparison with a quinine standard.

Results

The results of these measurements are presented in Table I and Fig. 1. The amino acid compositions are tabulated as per cent change from the

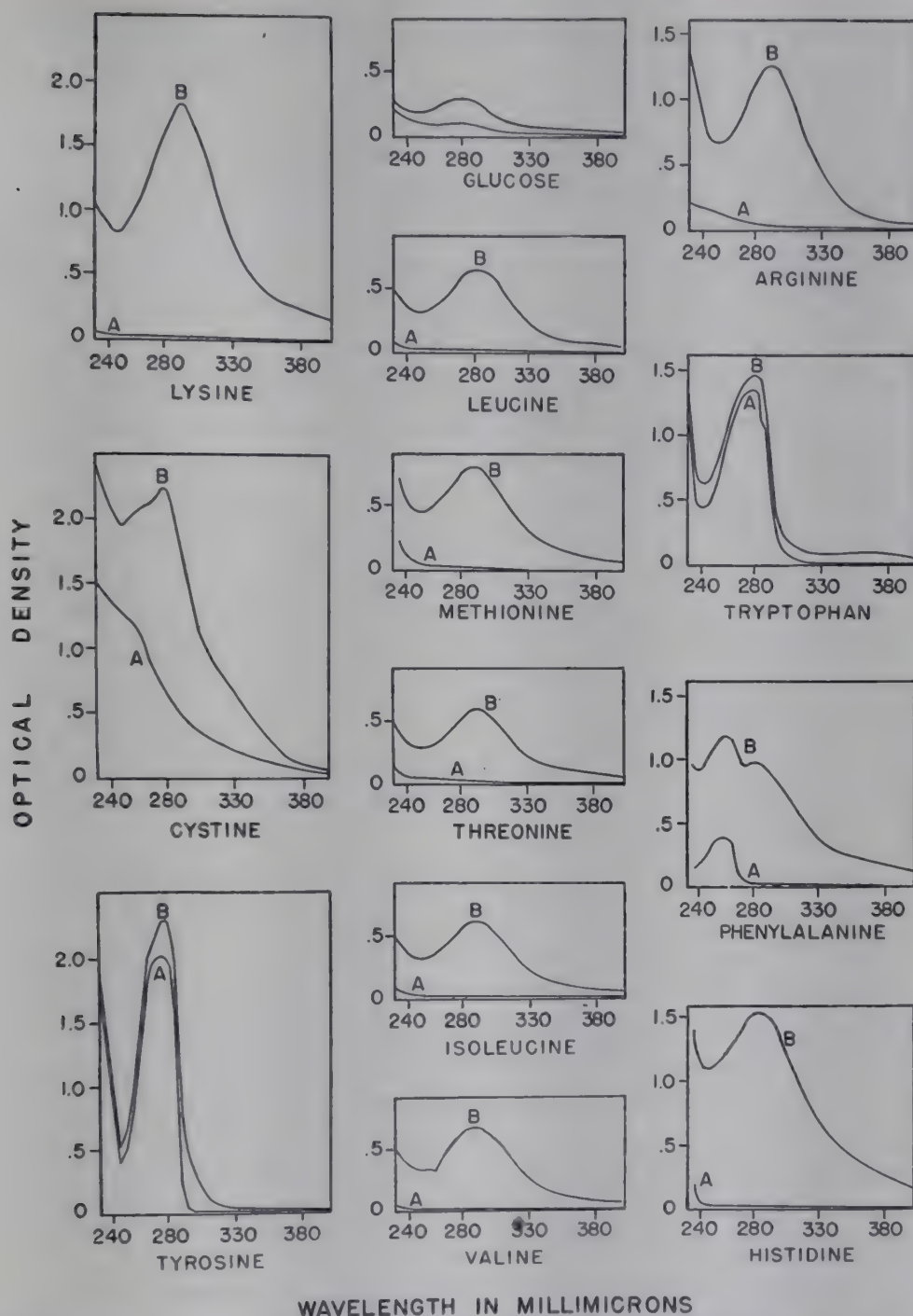


FIG. 1. Ultraviolet absorption curves; optical density *versus* wave-length. Curve A, 0.05 M solutions of amino acids, acetate buffer, pH 5.2, autoclaved 30 minutes and diluted 1:20. Curve B, 0.05 M solutions of amino acids in 0.25 M glucose, acetate buffer, pH 5.2, autoclaved 30 minutes and diluted 1:20. Cystine and tyrosine, at pH 2.1 and 2.5 respectively, autoclaved 30 minutes. Glucose, 0.25 M in acetate buffer, pH 5.2, autoclaved 30 and 120 minutes, respectively, diluted 1:20.

corresponding control without glucose. The color and degree of fluorescence for each solution are tabulated as optical density at 385 m μ and as per cent of the quinine standard, respectively. For the control solutions,

only the values after the 2 hour heating period are included, as they did not vary significantly from the values for the 0, 30, or 60 minute heating periods.

It is evident, as would be expected, that the amino acids are subject to increasing loss and increased development of color with the time of heating. However, the results of the fluorescence measurements are erratic and do not show a correlation with the time of treatment in every case. For example, in the case of tryptophan the fluorescence decreases regularly as the time of heating increases from 30 minutes to 2 hours, whereas with histidine the fluorescence increases and then decreases. Undoubtedly the highly increased color in the 2 hour heated samples serves to mask some of the fluorescence. This factor, however, does not appear to explain entirely the decreased fluorescence. For example, with threonine and isoleucine the intensity of color increases in the same way. For threonine the fluorescence continues to increase with time, but for isoleucine the fluorescence decreases as the heating is continued from 1 to 2 hours.

After heating for 2 hours, the concentrations, according to microbiological assay, of histidine, arginine, tryptophan, phenylalanine, lysine, and threonine are reduced by more than 20 per cent. The pattern of loss agrees well with results previously obtained with protein hydrolysate-glucose solutions except those for methionine, which was reduced to a greater degree in the protein hydrolysate (1).

It is apparent that the amino acid patterns for decreased concentration, relative color intensity (optical density at $385\text{ m}\mu$), and degree of fluorescence show very little correlation with each other. The difference in the patterns of these three measurements is well illustrated by lysine, which ranks fifth among the twelve amino acids in loss of microbiological potency, second in the amount of color produced, and eleventh in the amount of fluorescence. Although the values for fluorescence might have been different had it been possible to eliminate the interference of the intense color present in most of these solutions, it is not probable that the pattern of fluorescence measurements so obtained would have shown any better correlation with color or loss of amino acid content.

The ultraviolet absorption curves for each of the amino acids after 30 minutes autoclaving with and without glucose, and for glucose control solutions autoclaved for 30 minutes and 2 hours, are shown in Fig. 1. The characteristic absorption peak at $285\text{ m}\mu$ for glucose solutions heated alone developed slowly, the optical density after 2 hours being only 0.29 in contrast to the amino acid-glucose solutions which, after 30 minutes heating, showed characteristic absorption at $285\text{ m}\mu$ with a range of density from 0.60 to 2.3. Of the amino acid solutions without glucose, only tyrosine, tryptophan, cystine, and phenylalanine showed absorption in this range.

Heating with glucose led to an increase in absorption at $285\text{ m}\mu$ in every case.

Effect of Sodium Bisulfite on Amino Acid-Sugar Reaction—It is well known that sodium bisulfite will inhibit the color formation in this reaction (5). It was of interest to determine the effect of such an inhibitor upon the other characteristics of the reaction. When a 5 per cent protein hydrolysate-5 per cent glucose solution was heated in the presence

TABLE II
Effect of NaHSO_3 on Amino Acid-Glucose Reaction

	Solution I*	Solution II*	Solution III*
	Protein hydrolysate + NaHSO_3	Protein hydrolysate + glucose	Protein hydrolysate + glucose + NaHSO_3
	Loss of amino acids†		
	per cent	per cent	per cent
Arginine.....	4	36	30
Tryptophan.....	16	25	29
Lysine.....	9	23	15
Histidine.....	10	44	30
Threonine.....	5	10	13
Valine.....	3	10	6
	Physical measurements		
	Colorless	Brown	Colorless
Fluorescence‡.....	51	91	435
Optical density at $285\text{ m}\mu$	0.000§	0.475	0.252
“ “ “ $385\text{ m}\mu$	0.000§	0.093	0.009

* Autoclaved 1 hour at 15 pounds (121°).

† Method of Henderson and Snell (7).

‡ Expressed as per cent of the fluorescence of a standard solution containing 0.0005 mg. of quinine sulfate per ml. in $0.1\text{ N H}_2\text{SO}_4$.

§ Used as the blank in the Beckman DU spectrophotometer.

of 2 per cent sodium bisulfite, color formation was completely inhibited. In Fig. 2 it is seen that, although the absorption at $285\text{ m}\mu$ is less intense, it still occurs to a marked degree in the presence of the inhibitor. In Table II are given the results of microbiological assays for six of the amino acids in this heated protein hydrolysate-glucose solution. The values shown in Table II express per cent loss of each of the amino acids. Solution III, containing the inhibitor sodium bisulfite, showed losses of amino acids that were only slightly less than those of Solution II without sodium bisulfite. This demonstrated that the inhibitor has little or no effect in

preventing the reduction in microbiological potency of these amino acid solutions.

The fluorescence of the solution containing sodium bisulfite was approximately 5 times greater than that of the amino acid-glucose without bi-

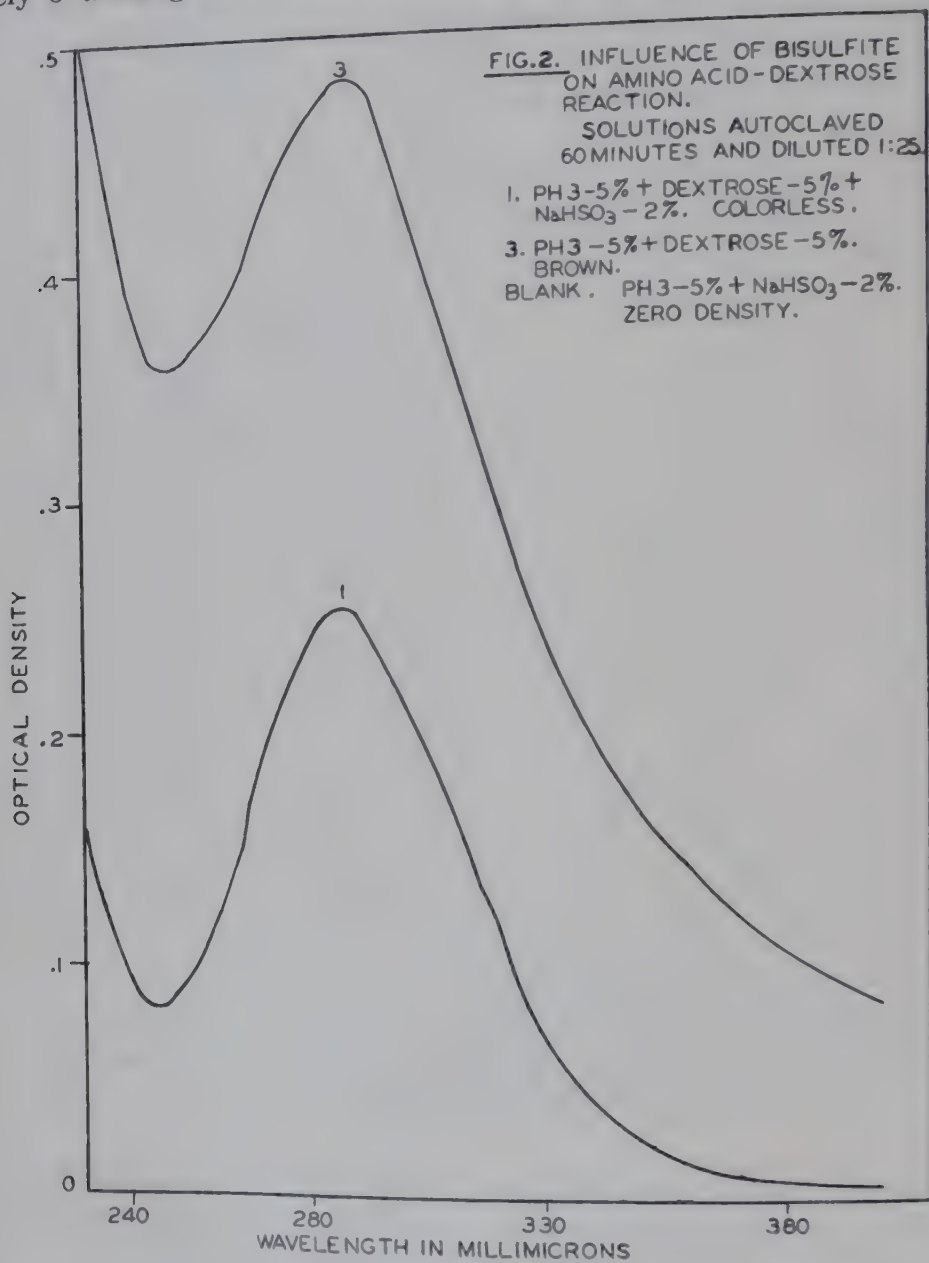


FIG. 2

sulfite. In the absence of the inhibitor very extensive color formation occurred. It was evident that strongly fluorescent compounds are produced which are completely colorless.

Separation of Fluorescent Colorless Reaction Products of Amino Acid-Sugar Reaction—To demonstrate further that the color and fluorescent

compounds are not identical, a qualitative separation was made by chromatography. An amino acid-glucose solution that was strongly colored as a result of heating was passed over a column of Decalso and washed several times with water. Upon elution with saturated KCl solution, a colorless and highly fluorescent fraction was obtained. It is thus possible to separate from the products of the amino acid-glucose reaction at least two fractions, one colorless and highly fluorescent, the other highly colored. It was not possible to obtain a colored fraction free of fluorescence.

Amino Acid-Glucose Reaction at Low Temperatures As Measured by Fluorescence—In Table III are presented the results of a storage experi-

TABLE III

Amino Acid-Glucose Reaction at Low Temperatures, As Measured by Fluorescence

Amino acid	Fluorescence, per cent quinine	
	10° (6 mos.)	25° (3 mos.)
Lysine.....	3	6
Methionine.....	2	2.5
Phenylalanine.....	2	13
Arginine.....	4	9
Cystine.....	2	2
Tryptophan.....	7	154*
Tyrosine.....	4	3
Valine.....	2	4
Histidine.....	3	45
Isoleucine.....	3	2.5
Leucine.....	2.5	3
Threonine.....	2.5	4
Mixture.....	4	75

* The solution was brown. A control solution, without glucose and also brown, had a fluorescence 6.5 per cent of quinine.

ment at lower temperatures. Aliquot portions of the amino acid and amino acid-sugar solutions described above were stored at 10° for 6 months and at 25° for 3 months, respectively. Except for tryptophan, the solutions remained colorless. Increases in fluorescence occurred only in the solutions of tryptophan, histidine, lysine, and phenylalanine stored at 25° in the presence of glucose.

It is clear then that the amino acid-glucose reaction is characterized by reduced microbiological availability of the amino acids, browning, production of fluorescent compounds, and characteristic ultraviolet absorption at 285 mμ.

Furthermore, the amino acid-sugar reaction can take place without the formation of color to form a complex that is highly fluorescent and

shows characteristic ultraviolet absorption, and that has a reduced biological value. The use of inhibitors of the browning reaction does not, therefore, give complete assurance of retention of nutritive quality.

SUMMARY

The course of the amino acid-glucose reaction has been studied in solutions of pure amino acids containing glucose by color development, ultraviolet absorption, fluorescence, and decrease in availability to microorganisms of certain amino acids, as measured by microbiological assay.

In amino acid solutions containing glucose, fluorescence developed slowly at 25° but was not measurable in solutions stored for 6 months at 10°.

The reaction products have characteristic ultraviolet absorption spectra with maxima in the region of 285 m μ and characteristic fluorescence.

There is little correlation between the decreased nutritional availability of the individual amino acids and the production of color or fluorescence in their solutions when heated with glucose.

The production of fluorescence, decreased availability of amino acids, and characteristic ultraviolet absorption were observed in the complete absence of color formation when sodium bisulfite was present. The data indicate that the fluorescent and colored reaction products are not identical. A qualitative separation of colorless fluorescent reaction products from the colored compounds produced in the reaction has been accomplished.

It is suggested that in the amino acid-sugar reaction the formation of colored compounds is preceded by the production of fluorescent reaction products. The amino acid-sugar reaction has important implications in practical nutrition. It is suggested that the measurement of color formation is an inadequate criterion for evaluating substances added for the purpose of inhibiting the reaction and that fluorescence may be a more useful tool in this respect.

The helpful suggestions and continued interest in this work of Dr. E. M. Nelson of the United States Food and Drug Administration, Washington, D. C., and Professor M. X. Sullivan of Georgetown University, Washington, D. C., are deeply appreciated.

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PROLINE AND HYDROXYPROLINE: PURIFICATION, REACTION WITH NINHYDRIN, AND SOME PROPERTIES OF THEIR N-NITROSO DERIVATIVES

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To standardize a new method¹ developed in this laboratory for the determination of the imino carboxyl nitrogen² of proline and hydroxyproline in the products of protein hydrolysates, without preliminary removal of amino or non-imino nitrogen, pure proline and hydroxyproline were necessary. All commercial samples tested were contaminated by measurable amounts of substances capable of evolving nitrogen gas in the Van Slyke nitrous acid procedure (2).

Because determination of proline and hydroxyproline imino carboxyl nitrogen by the ninhydrin-CO₂ technique (3) forms an integral part of the new method, and also because proline evolves CO₂ in excess of 1 mole per mole of imino acid on heating at 100° in the ninhydrin reaction for periods longer than 3.2 minutes (4), a reexamination of the reaction of both imino acids with ninhydrin (3, 4) was undertaken.

Preliminary treatment of amino acid mixtures with nitrous acid to convert all amino acids except proline and hydroxyproline to their respective α -hydroxy derivatives is employed in the method referred to above. While investigating the proper use of nitrous acid in this preliminary step, we discovered conditions for the quantitative conversion of proline and hydroxyproline to their respective N-nitroso derivatives, and some of the properties of these derivatives were investigated.

Analytical Procedure

α -Amino or α -Imino Carboxyl Nitrogen—Carboxyl nitrogen was determined by the method of Van Slyke, Dillon, MacFayden, and Hamilton (3). The reagents used in the manometric procedure were nearly saturated with NaCl, as recommended by MacFadyen (5), and aldehyde errors were minimized by the use of hydrazine, as suggested by Schott,

¹ Hamilton, P. B., and Ortiz, P. J., unpublished work.

² In conformity with the nomenclature of previous publications (Hamilton (1) p. 375 foot-note), the term imino carboxyl nitrogen is used to indicate values calculated as 1 gm. atom of nitrogen per mole of CO₂ evolved by reaction of the α -imino acids, proline and hydroxyproline, with ninhydrin.

Rockland, and Dunn (4), but with lactic acid solutions containing hydrazine (6). Ninhydrin was crystallized several times³ from 2 N HCl until 100 mg. of the purified reagent evolved no CO₂ on being heated for 20 minutes in a blank analysis at pH 2.5.

Octyl alcohol, used as an antifoaming agent, was distilled to remove substances capable of contributing CO₂ and tested in a blank analysis. Black alundum grains (No. 14 mesh), used to promote smooth boiling, contain ferrous iron which renders them unsuitable. White alundum grains were satisfactory after having been boiled for 2 hours in 6 N HCl, rinsed thoroughly with distilled water, and air-dried. Reactions were carried out at 100° in all-glass vessels (Hamilton and Van Slyke (7)) on 5 ml. volumes of solution with 100 mg. of ninhydrin at the pH values and for the times of heating mentioned in the experimental part. Unless otherwise specified, blank analyses were carried out with all the reagents included, and all analyses were made in duplicate.

Amino Nitrogen (Nitrous Acid Nitrogen)—Amino nitrogen was determined by the Van Slyke nitrous acid procedure (2) with a reaction time of 5 minutes.

Total (Kjeldahl) Nitrogen—Total nitrogen was determined by the macro-Kjeldahl procedure of Hiller, Plazin, and Van Slyke (8) with mercury as the catalyst and a digestion time of 6 hours.

EXPERIMENTAL

Purification and Properties of Proline and Hydroxyproline

Purification of Proline by Rhodanilate—Purification of L-proline was first attempted with rhodanilic acid, as recommended by Bergmann (9). The proline isolated was always slightly colored, was markedly hygroscopic, and developed a strong orange-yellow color when dissolved in warm ethanol, properties which are not those of freshly prepared pure proline and which apparently arise from contamination with traces of rhodanilic acid. Repeated crystallization reduced the yield to 50 per cent of the starting material without a satisfactory product having been obtained. Purification by means of the picrate was accordingly investigated.

Purification of Proline As Picrate—To proline dissolved in approximately 15 times its weight of glacial acetic acid, picric acid was added in slight excess over 1 mole, as recommended by Alexandroff (10); the solution was completed by warming. On adding 30 volumes of anhydrous diethyl ether, proline picrate rapidly separated; crystallization was completed in 2 to 3 hours at 4°. The picrate was dried *in vacuo* for 16 hours over calcium chloride (94 per cent). Four crystallizations were

³ Hamilton, P. B., and Ortiz, P. J., *Anal. Chem.*, in press.

often necessary to eliminate traces of substances capable of evolving nitrogen gas in the Van Slyke nitrous acid procedure and to obtain a product which gave theoretical yields of carboxyl nitrogen and had a melting point of 154° (corrected). These properties were unchanged by a fifth crystallization.

With each recrystallization, picric acid was added to provide approximately 0.005 mole excess. Otherwise dark brown crystals tended to form, and the amount of these increased with each subsequent crystallization.

The picrate was suspended in water, decomposed by means of aniline, and the free proline, isolated according to the method of Cox and King (11), was recrystallized from warm ethanol on the addition of an equal volume of anhydrous diethyl ether. In a typical experiment, the product of the fourth crystallization contained less than 0.05 per cent of its total nitrogen as amino nitrogen, gave a carboxyl nitrogen of 1.00 gm. atom per mole, and melted at 220.4° (corrected). A fifth crystallization did not alter these values.

*Analysis*⁴—

Calculated.	C 52.16, H 7.88, N (Dumas) 12.17
Found.	" 51.26, " 7.83, " " 12.02, ash 0.17

Freshly prepared proline was odorless, but within half an hour a characteristic musty smell was discernible. Fresh preparations of pure proline were not hygroscopic, since 100 mg., exposed for 3 weeks, showed no detectable change in weight. However, on prolonged exposure to air and light, it became hygroscopic and noticeably pink or brown in color. In an evacuated sealed glass ampul protected from light, it neither caked nor became discolored after 8 months storage. In general, the more crude the preparation, the more rapidly these changes took place. These findings appear to reconcile the observation of Towne (12) that his preparations were non-hygroscopic and that of Bergmann (9), who stated that his preparations were hygroscopic.

Hydroxyproline could be purified similarly by means of picric acid, but the yields were approximately 20 per cent lower because of the greater solubility of hydroxyproline picrate as compared with proline picrate in glacial acetic acid-ether mixtures. Pure hydroxyproline remained odorless and non-hygroscopic, but, when kept in loosely stoppered test-tubes, became noticeably pink in 8 months time, whether in light or in dark. No change was noted in samples placed in sealed evacuated ampuls and stored either in light or in darkness.

⁴ The authors wish to thank Dr. A. Elek of the Elek Micro Analytical Laboratories, 4763 West Adams Boulevard, Los Angeles 16, California, for the elementary analyses.

Recently, satisfactorily pure L-proline and hydroxy-L-proline have become available commercially.⁵

Reaction of Proline and Hydroxyproline with Ninhydrin

Proline or hydroxyproline was dissolved in water or hydrochloric acid solution adjusted to pH 1. For analysis at pH 2.5 or 4.7, 100 mg. of appropriate solid citrate buffer (3) were added to 5 ml. of the water solution. For analysis at pH 1, 5 ml. of the solution were employed with no buffer

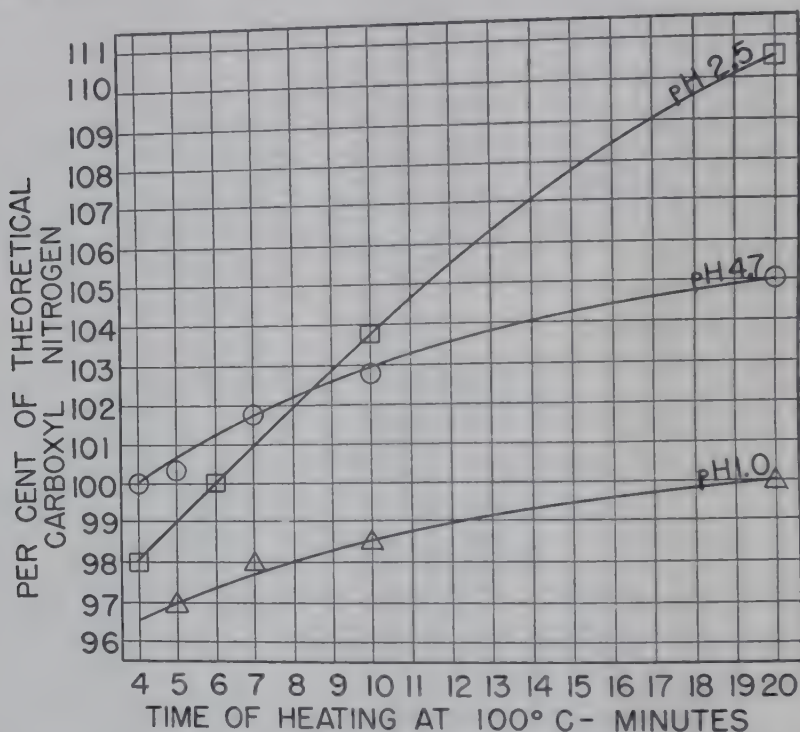


FIG. 1. Proline carboxyl nitrogen as a function of pH and time of heating at 100°. All reactions were carried out in triplicate on 5 ml. volumes of solution with 100 mg. of ninhydrin.

added. The vessels were charged with 100 mg. of ninhydrin and heated at 100° for the time indicated in Fig. 1.

Upon interpolation from Fig. 1, calculated theoretical carboxyl nitrogen values for proline were obtained on heating the vessels for 4 minutes at pH 4.7, 6 minutes at pH 2.5, and 20 minutes at pH 1.0. The apparent discrepancy between the 3.2 minutes heating at pH 2.5 found necessary by Schott, Rockland, and Dunn (4) to obtain theoretical carboxyl nitrogen values and the 6.0 minutes heating at the same pH reported in this paper resides in the fact that the former authors employed 50 mg. of ninhydrin per ml. of amino acid solution analyzed, whereas only 20 mg. of ninhydrin

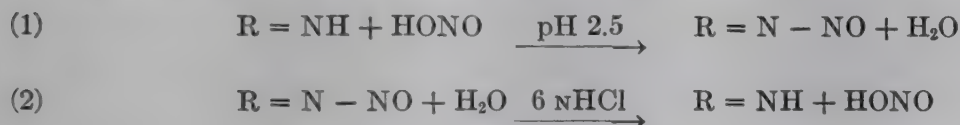
⁵ H. M. Chemical Company, 144 North Hayworth Avenue, Hollywood, California.

per ml. were employed in the present work. Both experiments are correctly reported ((3) p. 643).

Heating for 6 minutes at 100° at pH 4.7 evolved a greater excess of CO₂ than at pH 2.5, whereas, if heating was prolonged to 20 minutes at 100°, the excess CO₂ evolved was greater at pH 2.5 than at pH 4.7. No explanation can be offered for this effect. Hydroxyproline values are not shown, since CO₂ in excess of the calculated theoretical of 1 mole per mole of imino acid was never evolved.

N-Nitrosoproline and N-Nitrosohydroxyproline

As originally found by Van Slyke (2), neither imino acid yields nitrogen gas in the nitrous acid procedure, but a reaction does take place, since a product was readily extracted by means of diethyl ether after acidification to pH 1. Evaporation of the ether left a non-crystalline residue. This residue dissolved readily in 6 N HCl and, after 90 minutes boiling followed by evaporation, a white crystalline solid was obtained. Two recrystallizations from alcohol-ether yielded pure proline. By starting with 5 gm. of proline, 4.4 gm. (86 per cent) were recovered. These findings are compatible with the following equations:



Reasonably satisfactory evidence of the position of the NO group (Equation 1) was provided by the observation that the *N*-nitroso derivatives of proline or of hydroxyproline do not react with ninhydrin in the ninhydrin-CO₂ method. This would be probable only if the hydrogen of the imino nitrogen atom were replaced by an NO group. Reaction of α -amino acids with ninhydrin has been shown to be abolished ((3) p. 630) upon complete substitution of the nitrogen.

By using the reaction of Bratton and Marshall (13) for the detection of aryl amines, the formation of nitrous acid from *N*-nitrosoproline (Equation 2) was demonstrated. To a solution of *N*-nitrosoproline, solid ammonium sulfamate was added to decompose free nitrous acid, until frothing ceased on further additions. The solution was cooled and a few crystals of *p*-aminobenzoic acid and 1 mg. (knife point) of *N*-(1-naphthyl)-ethylenediamine were added; the solution was then acidified with 2 ml. of concentrated HCl. No color formed, indicating absence of free nitrous acid, but, on being gently warmed, the solution rapidly developed an intense purple color. Nitrous acid produced by acid hydrolysis of the *N*-nitrosoproline (Equation 2) reacted to form the diazotized aryl amine which combined with the coupling agent to form an azo dye.

This result supports the postulate of Hiller and Van Slyke (14) that proline and hydroxyproline exhibit reactions with nitrous acid that are typical and characteristic of secondary amines.

Formation of N-Nitrosoproline and N-Nitrosohydroxyproline—To prepare a solution of *N*-nitrosoproline, 50 mg. of proline were dissolved in 75 ml. of 0.3 N HCl, 3 gm. of solid NaNO_2 were added, and the flask was immersed in a boiling water bath (100°) for 5 minutes. The flask was then cooled and the solution diluted to 100 ml. with water. *N*-Nitrosohydroxyproline and blank solutions were prepared similarly.

Carboxyl Nitrogen of N-Nitrosoproline and N-Nitrosohydroxyproline—A 2 ml. portion of *N*-nitrosoproline solution and 2 ml. of water were placed in an all-glass reaction vessel, and 1 ml. of 60 per cent ammonium sulfamate solution (LaMotte, pure standard) was added slowly with cooling. With bromocresol green (0.04 per cent, aqueous) as an internal indicator, the reaction was adjusted to yellow-green by the dropwise addition of 5 N NaOH, followed by 1 N HCl. The solution was adjusted exactly to pH 2.5 by the addition of 100 mg. of solid citrate buffer (3) and analyzed for carboxyl nitrogen, with 100 mg. of ninhydrin and a heating time of 6 minutes. *N*-Nitrosohydroxyproline and blank solutions were analyzed similarly.

The *N*-nitroso derivatives of proline and hydroxyproline gave respectively 0.01 and 0.02 atoms of carboxyl nitrogen per mole, indicating that conversion of each imino acid to its respective *N*-nitroso derivative was at least 98 to 99 per cent complete.

Hydrolysis of N-Nitrosoimino Acids—A 5 ml. portion of *N*-nitrosoproline solution, 1.2 gm. of solid ammonium sulfamate, and 15 ml. of 8 N HCl were placed in a 100 ml. Kjeldahl flask and boiled vigorously. 6 N HCl was added as necessary, but fluid was allowed to boil away until 4 to 5 ml. remained after 90 minutes. *N*-Nitrosohydroxyproline and blank solutions were prepared similarly. The procedure effected the following reactions: *N*-Nitrosoproline was hydrolyzed to yield free proline and nitrous acid; nitric acid was reduced to nitrous acid; part of the nitrous acid reacted with ammonium sulfamate and part decomposed to oxides of nitrogen which escaped; ammonium sulfamate was hydrolyzed to ammonium sulfate.

The residual 4 to 5 ml. of hydrochloric acid were distilled off *in vacuo*, the dry residue was taken up in 5 ml. of water, again evaporated, and finally dissolved in 2 ml. of water. This solution was adjusted to pH 2.5 (approximately) and made up to 11.0 ml. with water. Carboxyl nitrogen was determined as described above.

Carboxyl nitrogens of the hydrolyzed *N*-nitrosoproline and *N*-nitrosohydroxyproline were found to be respectively 0.98 and 0.97 atoms per

mole, indicating practically quantitative reconversion of the *N*-nitroso derivatives to the free imino acids.

Extraction of N-Nitrosoimino Acids by Means of Ether—A 5 ml. sample of the *N*-nitrosoproline solution, 1 ml. of 60 per cent ammonium sulfate, and 1 ml. of 6 M phosphoric acid were placed in a glass thimble and extracted with ether in a Soxhlet apparatus as described by Gould (15). The receiving flask was charged with 10 ml. of 6 N HCl and 250 ml. of diethyl ether. *N*-Nitrosoproline solution was extracted for 4 hours, the *N*-nitrosohydroxyproline solution for 16 hours.

After extraction, ether was evaporated and the residual acid solution in the receiving flask transferred to a Kjeldahl flask with 6 N HCl. Hydrolysis, removal of excess acid, neutralization, and analysis for carboxyl nitrogen were carried out as described previously.

Recovery of proline and hydroxyproline carboxyl nitrogen were 0.98 and 0.97 atoms per mole respectively. The carboxyl nitrogen lost could not be demonstrated in the extracted residue in the thimble. Apparently, destruction of small amounts of imino acids took place. This is not unexpected, as solutions of *N*-nitrosoproline or *N*-nitrosohydroxyproline containing excess nitrous acid lose 52 and 39 per cent respectively of their original carboxyl nitrogen on standing for 4 weeks at room temperature.

Extraction of N-Nitrosoproline and N-Nitrosohydroxyproline As Function of pH—Above pH 4.0, neither *N*-nitrosoproline nor *N*-nitrosohydroxyproline could be extracted from an aqueous solution by ether. At lower pH values, the *N*-nitroso derivatives were extracted, the amount becoming maximal at approximately pH 1.

Extraction of N-Nitrosoproline from Acid Aqueous Solution by Organic Solvents—10 ml. portions of a solution of *N*-nitrosoproline acidified to pH 1 were shaken with 60 ml. portions of methyl acetate, ethyl acetate, *n*-propyl acetate, isopropyl acetate, methylethyl ketone, or methylal. 50 ml. of solvent were removed and proline carboxyl nitrogen was determined. These solvents extracted 1.5 to 1.7 times as much *N*-nitrosoproline as diethyl ether. In a similar experiment, only a third as much *N*-nitrosohydroxyproline as *N*-nitrosoproline was extracted by ether. The ability of solvents other than ether to extract *N*-nitrosohydroxyproline was not investigated. Diethyl ether was considered the solvent of choice because of its low boiling point and ease of removal, and because it contributes nothing to a blank analysis.

DISCUSSION

The discrepancy in statements in the literature regarding hygroscopic properties of proline appears to be reconciled by the observations described. Preparations of proline can apparently be kept unaltered for

only a relatively short time when exposed to light and air. Proline is pure white and is not hygroscopic at first but may become hygroscopic and discolored in time. Storage in evacuated sealed glass ampuls protected from light seems to be the most satisfactory way of preventing change.

The finding of Schott, Rockland, and Dunn (4) that proline evolves CO_2 in excess of 1 mole per mole of imino acid on continued heating in the ninhydrin analysis is confirmed. Hydroxyproline, on the other hand, does not evolve more than 1 mole of CO_2 per mole of imino acid.

Application of the principles described makes possible quantitative methods for the determination of total imino carboxyl nitrogen in protein hydrolysates and these will be described in a subsequent publication. Also, since proline or hydroxyproline carboxyl nitrogen can be determined after reaction with nitrous acid, either in the reaction mixture or after extraction of the *N*-nitroso derivative by ether, procedures are provided whereby purity of these imino acids may be checked. A single procedure can therefore be substituted for separate determinations of carboxyl nitrogen (3) and amino nitrogen (2). For conditions outlined in previous sections of this paper, proline or hydroxyproline nitrogen values are multiplied by 1.02 or 1.03 respectively to correct for loss of carboxyl nitrogen. These losses are sufficiently constant and reproducible so that, for pure imino acids, corrected values are within ± 1 per cent of theoretical.

A single attempt to isolate proline and hydroxyproline from a hydrochloric acid hydrolysate of gelatin by means of ether extraction of the *N*-nitroso derivatives gave low yields, as the experimental conditions selected were not ideal.

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SUMMARY

1. Purification of proline or hydroxyproline by means of the picrate is described. Proline is a white, crystalline, non-hygroscopic solid with a characteristic odor when freshly prepared. Unless stored with protection from air and light, it becomes caked, discolored, and markedly hygroscopic in time. Hydroxyproline discolors but does not become hygroscopic.

2. The finding of Schott, Rockland, and Dunn (4) that proline evolves

carboxyl nitrogen in excess of 1 gm. atom per mole in the ninhydrin reaction is confirmed. Theoretical yields of CO_2 are evolved from proline after 6 minutes at 100° with 20 mg. of ninhydrin per ml. at pH 2.5 in the ninhydrin method (3). At pH 4.7 and the same concentration of ninhydrin, theoretical yields of CO_2 are obtained after 4 minutes at 100° .

3. Proline and hydroxyproline, when heated at 100° in solutions of approximately pH 2.5 containing nitrous acid, are converted quantitatively to their respective *N*-nitroso derivatives. Hydrolysis of the *N*-nitroso derivatives yields 98 and 97 per cent respectively of the original acid, as measured by the recovery of imino carboxyl nitrogen.

4. *N*-Nitrosoproline and *N*-nitrosohydroxyproline can be extracted from acid aqueous solutions (below pH 4.0) by various organic solvents. The conditions are outlined whereby 98 and 97 per cent respectively of each imino acid can be recovered by extraction with ether.

5. Procedures taking advantage of the properties of proline and hydroxyproline and their *N*-nitroso derivatives are indicated whereby these imino acids may be determined in acid protein hydrolysates, their purity may be checked, and they may be isolated from protein hydrolysates.

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THE EFFECT OF INSULIN AND ADRENAL CORTICAL EXTRACT ON THE HEXOKINASE REACTION IN EXTRACTS OF MUSCLE FROM DEPANCREATIZED CATS*

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The search for the locus of the chemical action of insulin continues with unabated zeal in a dozen laboratories in this country and abroad. Striking effects of insulin upon metabolic reactions in the intact animal or in surviving organs can be demonstrated with the greatest of ease, but the great advances in enzyme chemistry within recent years have given rise to the expectation that similar effects could be demonstrated in homogeneous systems of tissue enzymes completely free of cellular organization.

Antithetical to this possibility is the concept that the structural integrity of the cell is required for the action of insulin. A decision between these two hypotheses is of prime importance. Because, depending upon which is true, the search for the fundamental mechanisms governing the action of the hormone would diverge quickly, since the chemical and physical mechanisms which are operative in homogeneous as opposed to non-homogeneous systems are strikingly different.

For these reasons a demonstration of any action of insulin in any homogeneous system becomes of major importance.

Recently interest has centered about the hexokinase reaction in muscle and brain extract. Price, Cori, and Colowick (1) reported that this reaction was inhibited by injecting rats with anterior pituitary extract (APE) prior to the preparation of the muscle extract, or by adding APE to the enzyme preparation *in vitro*. Preparations from alloxanized rats showed inhibited activity similar to that induced by the injection of APE. All cases of APE inhibition were released by insulin which by itself did not enhance the hexokinase activity. Price, Slein, Colowick, and Cori (2) further reported that adrenal cortical extract (ACE) had no effect on the hexokinase reaction in normal muscle extract, but greatly intensified the inhibitory effect of added or previously injected APE. In cases of alloxanized rats ACE alone produced a marked inhibition, invariably released by insulin.

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Colowick, Cori, and Slein (3) reported further that the hexokinase activity of normal rat muscle or beef brain extracts was not influenced by ACE but was inhibited by protein fractions of the anterior pituitary. They also found that the hexokinase activity of extracts of muscles from alloxanized rats was inhibited by ACE. In all cases the inhibition, when it occurred, was relieved by insulin. The authors stressed the extreme lability of the inhibitory factor. This was manifested in two ways: (1) by an initial lag of the hexokinase reaction which was relieved as the inhibitory factor was destroyed with time; (2) by the complete destruction of the inhibitory factor upon aging the extract for a period of 45 to 120 minutes at 0°. Broh-Kahn and Mirsky (4) reported that the hexokinase activity of rat muscle extracts from alloxanized rats showed values comparable to those from normal rats. The addition of insulin had no action on either the normal or diabetic extract. They also studied the action of potent diabetogenic factors obtained from the anterior pituitary. On occasion they could demonstrate inhibition of the hexokinase reaction upon the addition of such extracts and a reversal by insulin. But the effect was inconstant and in no wise related to the diabetogenic activity of the preparation.

Stadie and Haugaard (5) reported that the diabetic state induced by alloxan did not alter the rate of the hexokinase reaction when compared to that of a control group of rats. They were unable to show that adrenal cortical extract (ACE) with or without insulin had any effect whatsoever upon the rate of the reaction. In a systematic study they were unable to show that there was any initial slow phase of the hexokinase reaction either in muscle or kidney extract of normal or alloxanized rats. The summation of the work of these authors failed to reveal any interrelation between insulin, ACE, and the hexokinase reaction in muscle extracts of normal or alloxanized diabetic rats. Reid, Smith, and Young (6) reported that "in a number of instances" they confirmed Cori's findings of the reversal by insulin of hexokinase inhibition by APE. Many pituitary preparations of highly diabetogenic character were reported by them to show no insulin-reversible inhibition of hexokinase activity. In a later report from the same laboratories Smith (7) stated that of a series of twelve rats made diabetic by treatment with alloxan only one yielded a muscle extract whose activity was increased by insulin *in vitro*. Christensen, Plimpton, and Ball (8) prepared hexokinase from red cells of normal and alloxanized diabetic rats. They made a systematic study of the influence of insulin, adrenal cortical hormone, and of the diabetic or hypophysectomized state upon the rate of the reaction. They were unable to show that these factors played any significant rôle in the reaction.

Since this work was done with tissues from normal and alloxan-diabetic rats, it was possible that the variation in the diabetic state might, in part at least, be responsible for the variability of the results obtained by different

investigators. For this reason chiefly we undertook studies of the hexokinase reaction, using extracts prepared from the muscles of depancreatized cats. The system used was precisely the same as that used in our reported studies on rat muscle extract, *viz.* muscle extract, glucose, adenosine triphosphate (ATP), fluoride, in which the rate of disappearance of glucose was measured anaerobically. These experiments together with the weight of the evidence already cited fail to support the concept that insulin alone or in combination with pituitary or adrenal factors has any influence upon the hexokinase reaction in homogeneous enzyme systems from mammalian muscle.

Our early experiments with cat muscle extract were quite puzzling but a pattern quickly became apparent, which made the behavior of the system understandable. The pertinent major facts observed were as follows: (1) freshly prepared extracts of cat muscle, unlike rat muscle extracts, contained large amounts of glycogen; (2) in most instances the *apparent* hexokinase activity of cat muscle extract, when calculated from the rate of disappearance of glucose, was very low; (3) on the other hand, if measured by the rate of formation of phosphate ester, the hexokinase reaction appeared to be quite rapid. It was soon found that this paradox could be explained by the fact that the glycogen in the extract decomposed by two reactions giving rise to products which resulted in erroneous estimations of the hexokinase reaction. The first is an amylytic reaction producing reducing substances (containing no phosphate) which are quantitatively measured by methods used for the determination of glucose. In consequence the decrease of glucose by the hexokinase reaction and the increase of reducing substances by this amylytic reaction cancel each other to a greater or less extent, thus accounting for the apparent low hexokinase reaction. The second reaction is a phosphorylation of glycogen which together with the hexokinase reaction contributes to the formation of phosphate esters. Measurement of phosphate ester formation by the customary equation

$$\Delta \text{ ester} = -[\Delta \text{Pi} + \Delta \text{P}_i]$$

accordingly gives an apparent hexokinase reaction much enhanced over the actual.

It is obvious that these characteristics of fresh cat muscle extract (as customarily prepared) make it impossible to obtain reliable measurements of the hexokinase reaction. However, as will be shown later, we were able to overcome the difficulty and thus secure valid measurements of hexokinase activity in muscle extracts from depancreatized cats.

Methods

Muscle Extracts—The cats were used 48 hours after total pancreatectomy. After pentobarbital anesthesia the muscles of the hind legs were rapidly ex-

cised and collected on ice. They were cut into small pieces by scissors and ground in a mortar with a small amount of sand and the slow addition of 1 to 2 volumes of ice-cold water. The resulting paste was centrifuged at 4000 r.p.m. and the supernatant collected. A second extract was prepared by grinding the residue with 1 volume of ice water and centrifuging as before.

Assay for Hexokinase Activity—The reaction system had the following composition: 0.038 M NaHCO_3 , 0.020 M NaF , 0.006 M ATP, 0.0042 M glucose, and 0.008 M MgCl_2 , and 2.8 ml. of muscle extract. Total volume, 6.0 ml. Adrenal cortical extract (Upjohn) when present, 0.033 ml. per ml. of reaction mixture. Insulin (Lilly amorphous) when present, 1 unit per ml. The components of the reaction mixture were added to vessels cooled to 0° and an initial 1 ml. sample taken for analysis. The vessels were then gassed with a mixture of 95 per cent nitrogen and 5 per cent CO_2 and equilibrated with shaking at 30° . Samples were taken at various times for analysis of reducing substances. From the data the regression coefficient together with its standard error was calculated.

Breakdown of Glycogen—A number of experiments were designed to study the nature of the breakdown products obtained from the glycogen originally present in the muscle extracts. In these experiments glycogen disappearance, changes in inorganic and acid-labile phosphate, and appearance of reducing substances were determined. ATP and glucose were usually omitted from the reaction mixtures.

Chemical Methods—Reducing substances were determined by the method of Miller and Van Slyke (9) after deproteinization of the samples by ZnSO_4 and Ba(OH)_2 . Glycogen was determined by the method of Good, Kramer, and Somogyi (10). Inorganic phosphate and phosphate hydrolyzed after heating to 100° in 1 N HCl for 7 minutes were determined on samples deproteinized with trichloroacetic acid.

Results

When the hexokinase reaction was measured in cat muscle extract by the determination of the rate of disappearance of reducing substance (presumed to be glucose), low activities were generally obtained. In experiments in which freshly prepared muscle extracts were used an initial slow rate of reaction was observed, followed by a significant increase in activity after 10 to 20 minutes. Fig. 1, Curve A is a representative case of such an experiment. As will be subsequently shown, the initial lag is correctly explained by the formation of reducing substances from glycogen which partially cancel out the disappearance of glucose by the hexokinase reaction. But the rate of formation of reducing substances from glycogen gradually falls off and finally ceases. There is in consequence an apparent increase

in the rate of the hexokinase reaction. This interpretation is well supported by the observation that when the muscle extract is maintained for 1 hour at 30° the glycogen is completely decomposed. The hexokinase activity therefore is greater than with the fresh extract and shows no initial lag (Fig. 1, Curve B). Without knowledge of the rôle of glycogen this contrasting behavior of fresh and aged extracts could have been interpreted as being due to the destruction by aging of a labile inhibitory factor of hormonal origin.

REDUCING SUBSTANCES
AS GLUCOSE
MICROMOLES / ml. EXTRACT.

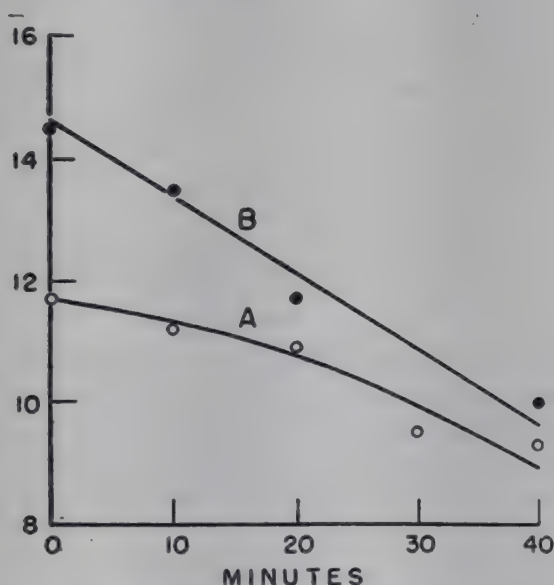


FIG. 1. Hexokinase activity of muscle extract of depancreatized cat. Reaction system, 0.03 M NaHCO_3 , 0.02 M NaF , 0.01 M MgCl_2 , 0.006 M ATP, 0.004 M glucose; 2.4 ml. of muscle extract; total volume 5.2 ml.; gas phase 95 per cent N_2 -5 per cent CO_2 . Curve A, extract freshly prepared. Curve B, same extract after equilibration at 30° for 2 hours.

The behavior of the system becomes clearer when ATP and glucose are omitted from the system. We see from the data shown in Fig. 2 that (1) a fresh extract (Curve A) shows a considerable increase in reducing substance with time; (2) the extract aged for 2 hours at 30° (Curve B) shows a negligible rate of increase of reducing substances; (3) when glycogen is added to the aged extract (Curve C), the original observation of an increase of reducing substance is found. It is apparent that glycogen in fresh muscle extract gives rise to reducing substances which are not precipitable by zinc sulfate-barium hydroxide and therefore non-phosphate ester in character.

A typical experiment which shows more exactly the reactions involving glycogen in cat muscle extract is shown in Table I. The reaction mixture

contained muscle extract, NaHCO_3 , NaF , and MgCl_2 . The fresh extract contained initially a large amount of glycogen ($30\text{ }\mu\text{M}$ per ml. of extract).

REDUCING SUBSTANCES
AS GLUCOSE
MICROMOLES/ml. EXTRACT.

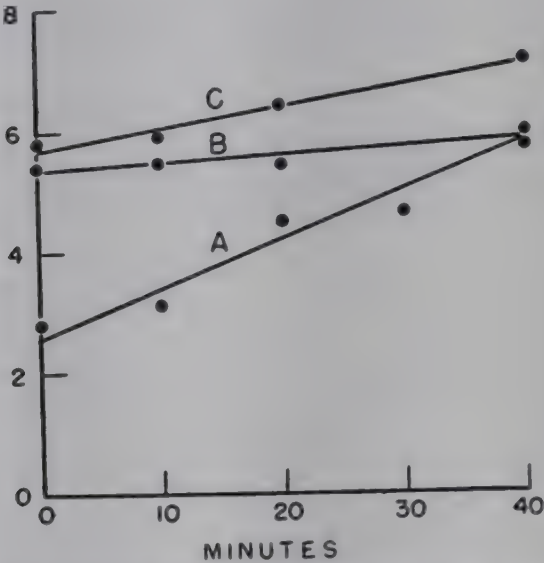


FIG. 2. Production of reducing substances in cat muscle extract. The extracts and the reaction system are the same as in the experiment illustrated in Fig. 1, except that ATP and glucose have been omitted. Curve A, extract freshly prepared. Curve B, same extract after equilibration at 30° for 2 hours. Curve C, the aged extract after addition of glycogen ($10\text{ }\mu\text{M}$ of glucose equivalents per ml. of extract).

TABLE I

Decomposition of Glycogen in Cat Muscle Extracts

Reaction system, 0.038 M NaHCO_3 , 0.020 M NaF , 0.007 M MgCl_2 ; total volume 36 ml.; freshly prepared cat muscle extract 17 ml.; initial glycogen content $30\text{ }\mu\text{M}$ per ml. Gas phase 95 per cent N_2 -5 per cent CO_2 ; temperature 30° . Glycogen and reducing substances are expressed in micromoles of glucose equivalents per ml. of extract. Phosphate ester is expressed as micromoles per ml. of extract.

Time	Glycogen decrease	Phosphate esters increase	Reducing substance	
			Before hydrolysis increase	After hydrolysis increase
min. 0-60	-16.6	+10.9	+5.8	+10.4

The following changes are to be noted: (1) a rapid disappearance of glycogen; (2) phosphate ester formation (measured by decrease of inorganic phosphate); (3) increase of reducing substances in samples deproteinized by zinc sulfate-barium hydroxide. These reducing substances are not phos-

phorylated intermediates, since control experiments show that only traces of phosphate are found in such filtrates. (4) When these deproteinized samples were subjected to hydrolysis at 100° in normal acid for 3 hours, an increase of reducing value was found. This indicates the presence of polysaccharides other than glycogen in the filtrates. (Most of the glycogen is carried down by $\text{ZnSO}_4\text{-Ba(OH)}_2$ precipitation; a correction was made for the small amounts found in the filtrates.) (5) Most of the reducing substance present after hydrolysis was fermented by bakers' yeast, indicating that in all probability it was glucose derived from the glycogen (data not included in Table I).

For the purpose for which these experiments were performed a precise interpretation of balance need not be carried too far. The experiments demonstrate definitely that cat muscle extract contains glycogen which forms hydrolyzable, fermentable, reducing polysaccharides, and in addition phosphate esters. These reactions make the customary methods for measuring the hexokinase reaction in muscle extract invalid.

The question of the origin of the amylotic enzyme in the muscle extract has not been studied systematically. It is probably partly introduced by the blood invariably present in muscle extract as ordinarily prepared. Cat plasma was found to cause a rapid amylotic breakdown of glycogen. However, the presence of amylase in muscle proper cannot be excluded, since it was not found possible to decrease significantly the amylotic action of muscle extract by a preliminary perfusion of the cat muscle with saline to eliminate the blood.

Effect of Glycogen on Measurement of Rate of Hexokinase Reaction

The data in Table II illustrate more specifically how the presence of glycogen in muscle extract renders the customary method for measuring hexokinase activity invalid. Rat muscle was selected to show that the phenomena are not unique for cat muscle. As we have stated before, our rat muscle extracts contain a negligible amount of glycogen initially. Control experiments show no spontaneous formation of reducing substances or phosphate esters. Hence, decrease (Experiments A, Table II) of reducing substances and phosphate ester formation becomes a true measure of the rate of formation of glucose-6-phosphate. On the other hand, when a small amount of glycogen is added initially (as is the case in Experiments B of Table II), the measurement of the reducing substance gives an apparent rate of hexokinase reaction which is approximately half of the true value. If the rate of phosphate ester formation is measured, a result approximately $2\frac{1}{2}$ times that of the true rate is obtained. These findings were explained satisfactorily, as in the case of the cat muscle extract, by demonstrating in separate experiments in the absence of ATP and glucose (not shown in

Table II) that formation of reducing substance from glycogen occurred together with phosphate ester formation.

In our experience these difficulties do not occur spontaneously when rat muscle extracts are used because insignificant amounts of glycogen are present. However, variability of strain of rat, state of nutrition, methods of preparation, etc., might result in rat muscle extracts containing appreciable quantities of glycogen. In such instances, serious misinterpretation of

TABLE II

Hexokinase Activity of Rat Muscle Extract in Presence and Absence of Glycogen

System, 2.4 ml. of 1:3 water extract of rat muscle; total volume 5.0 ml.; 0.040 M NaHCO₃, 0.023 M NaF, 0.006 M ATP, 0.004 M glucose, 0.010 M MgCl₂. Glycogen added in Experiments B. Gas phase 95 per cent N₂-5 per cent CO₂. Glycogen and reducing substances are expressed as micromoles of glucose equivalents per ml. of extract. Inorganic phosphate and phosphate esters are expressed as micromoles per ml. of extract. Each experiment done in duplicate. Time 30 minutes; temperature 30°.

Experiment No.	Changes		
	Glycogen	Reducing substances	Phosphate esters
A1	0	-4.3	+6.5
A2	+0.1	-4.4	+6.3
B1	-5.0	-2.7	+14.4
B2	-5.3	-2.2	+14.4

TABLE III

Initial Glycogen Content and Appearance of Reducing Substance in First and Second Extracts of Cat Muscle

Extract	Initial glycogen, μ M glucose per ml.	Δ reducing substance, μ M per ml. per 40 min.
1st.....	23.0	+3.0
2nd.....	3.0	0.0

experimental data could easily arise, unless the rôle of glycogen as a source of error is appreciated.

Elimination of Error Due to Glycogen in Measuring Hexokinase Reaction

The simplest way to eliminate the error is to prepare a muscle extract without glycogen. It was found that if the minced cat muscle was ground once with water most of the glycogen was removed in this first extract. A second extract possessing considerable hexokinase activity could then be prepared containing little or no glycogen and giving rise to negligible amounts of reducing substances. The data of a representative control experiment of this type are shown in Table III.

A technique was then worked out by which the muscle mince was extracted twice with water. Care was taken to employ the utmost speed in the preparative steps and to maintain the temperature close to 0°. The average time of preparation was 20 minutes from the time of removal of the muscle from the cat to the beginning of the measurements at 30°.

Effects of Hormones on Rate of Hexokinase Reaction in Cat Muscle Extracts

By this technique five "second extracts" were obtained from depancreatized cats. It is our conviction that measurements on them gave a true comparative measurement of the rate of the hexokinase reaction. The data are given in Table IV. The reaction mixture was equilibrated

TABLE IV

Effect of Hormones on Rate of Hexokinase Reaction in Extracts of Muscle from Depancreatized Cats

Muscle mince washed once to remove glycogen; extract made from washed mince. System, 2.8 ml. of muscle extract, 0.040 M NaHCO_3 , 0.023 M NaF, 0.006 M ATP, 0.004 M glucose, and 0.010 M MgCl_2 . Total volume 6.0 ml.; gas phase 95 per cent N_2 -5 per cent CO_2 . Rate of reaction determined from glucose measured at 0, 10, 25, and 35 minutes and expressed as $1000 \times$ micromoles per ml. of extract per minute $\pm$ s.e. of regression. Insulin and adrenal cortical extract (ACE) added as indicated.

Cat No.	Rate of reaction			
	None	Insulin	ACE	ACE + insulin
1	69 $\pm$ 14	66 $\pm$ 6	46 $\pm$ 8	79 $\pm$ 12
2	51 $\pm$ 7	37 $\pm$ 10	30 $\pm$ 17	31 $\pm$ 2
3	76 $\pm$ 3	75 $\pm$ 4	66 $\pm$ 8	62 $\pm$ 4
4	107 $\pm$ 1	130 $\pm$ 5	101 $\pm$ 13	125 $\pm$ 8
5	55 $\pm$ 12	59 $\pm$ 8	62 $\pm$ 17	57 $\pm$ 5

at 30° and samples removed for analysis of total reducing substances at 0, 10, 25, and 35 minutes. From the data the regression coefficient together with its standard error was calculated. This gives the rate of decrease of glucose, *i.e.* the hexokinase reaction. This was determined in the absence of added hormones, in the presence of insulin, adrenal cortical extract, and adrenal cortical extract plus insulin. In all cases (data not given) analyses of glycogen were carried out, and it was shown by appropriate control experiments that the appearance of reducing substances from glycogen was not a source of error in the calculation of the hexokinase reaction. The hexokinase rates observed are of the order of that observed by us for rat extracts. There is no indication from our data, when plotted, of a time-course of reaction other than rectilinear during the period of observation. The consistency of the data is also indicated by the low value, in most instances, of the standard error of the regression coefficient.

The data show that the addition of insulin, of ACE, or ACE plus insulin has no influence whatever upon the rate of the reaction. This conclusion is in conformity with that made from our own data obtained in the case of alloxanized diabetic rats.

We wish to express our appreciation to Phyllis Stapley Tuddenham and Mary-Ellen Miller for their expert technical assistance.

SUMMARY

1. The hexokinase reaction has been studied in muscle extracts from depancreatized cats.
2. A considerable amount of glycogen is present initially in fresh cat muscle extract. The glycogen was found to undergo two reactions both giving rise to decomposition products interfering with the usual methods of measuring the hexokinase reaction: (1) an amylotic breakdown product giving rise to non-phosphorylated reducing substances; (2) a phosphorylative reaction giving rise to phosphate esters.
3. Valid measurements of the rate of the hexokinase reaction were obtained in extracts of muscle from depancreatized cats from which most of the glycogen had been removed by a preliminary extraction with water.
4. No effects of adrenal cortical extract or insulin singly or together on the hexokinase reaction were observed in such extracts.

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A MICROMETHOD FOR THE DETERMINATION OF XANTHINE AND GUANINE IN URINE*

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In the course of investigations involving purine metabolism, it became necessary to obtain a rapid and accurate method for the estimation of various purine bases in small amounts of urine. Existing methods for determining purine bases, *e.g.* those of Krüger and Schmidt (1) and Hunter and Givens (2), require such large amounts of urine that they could not be used. Attempts to apply Hitchings' method (3) for xanthine and guanine in tissue extracts to urine failed in that recovery values of added xanthine ranged from 10 to 300 per cent.

The following method for the determination of xanthine and guanine in urine is both rapid and sensitive compared to other methods and consistently gives recovery values of 90 to 100 per cent.

EXPERIMENTAL

The method involves precipitation of all purine bases present in the urine sample with an ammoniacal cupric sulfate reagent in the presence of excess glucose and measurement of xanthine and guanine colorimetrically after dissolving the copper-purine precipitate in acid.

Reagents—

1 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ plus 5 ml. of concentrated ammonium hydroxide. Dilute to 100 ml.

10 per cent glucose solution.

5 N hydrochloric acid.

1 per cent potassium ferricyanide.

Saturated sodium carbonate solution.

Folin phenol reagent with lithium sulfate (4).

Xanthine standard containing 0.4 mg. per ml. at pH 7.

*Procedure—*To 4 ml. of undiluted neutralized urine in a 15 ml. centrifuge tube calibrated at 5 ml. are added 4 ml. of the ammoniacal copper reagent and 1 ml. of the glucose solution. The solution is mixed, placed in a boiling water bath for 10 minutes, and then cooled with tap water. If many sam-

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ples are to be analyzed, it is convenient to put the tubes in a metal test-tube rack which can be placed in a pan of vigorously boiling water.

After cooling, the heavy flocculent precipitate of the copper-purine salts is centrifuged until clear (5 to 10 minutes). The precipitate should be washed once or twice with water or until the blue color of ammoniacal cupric sulfate disappears. 1 ml. of 5 N hydrochloric acid is added and the tubes are made up to 5 ml. with water. The tubes are heated in the water bath for about 5 minutes in order to dissolve the copper-purine salts. At this point, the solution may be slightly cloudy because of non-purine, acid-insoluble sediments originally present in the urine.

Next, 0.5 ml. of 1 per cent potassium ferricyanide is pipetted into each tube; the solutions are mixed and centrifuged for 10 minutes. This treatment with ferricyanide removes the last traces of copper which might interfere in the colorimetric estimation of xanthine and guanine.

After decanting the supernatant solution from the ferricyanide precipitate, 2 ml. of the supernatant liquid are mixed with 1 ml. of the Folin phenol reagent, followed by 5 ml. of saturated sodium carbonate. Color develops immediately and remains stable for several hours. The colored solutions are diluted to 20 ml. and read in an Evelyn colorimeter with a 660 $m\mu$ filter.

A standard curve is obtained by using either xanthine or guanine as a standard. The series of standards is carried through the foregoing procedure from the point of addition of 1 ml. of 5 N hydrochloric acid, since the addition of yellow ferricyanide to the unknown sample necessitates adding an equivalent amount to the standards.

By a simple modification of dilutions and standards this procedure can be adapted for use with any type of colorimeter.

Results

A series of purines was carried through the foregoing procedure in order to observe whether any purine except xanthine and guanine gives a positive color reaction. The substances tested, besides xanthine and guanine, were adenine, uric acid, and hypoxanthine. In addition, standards of allantoin and uracil were tested by the same procedure. The results of these experiments are given in Fig. 1. The abscissa denotes the purine content of the standard before the procedure was begun. These standards were carried through the entire procedure given above, including the precipitation. The results demonstrate that, although slight color development is obtained with the other purines, this slight discrepancy may be due to impurities of xanthine and guanine held tenaciously even after recrystallization. Uracil, a pyrimidine, and allantoin are not precipitated in the procedure. Guanine and xanthine appear to give similar color develop-

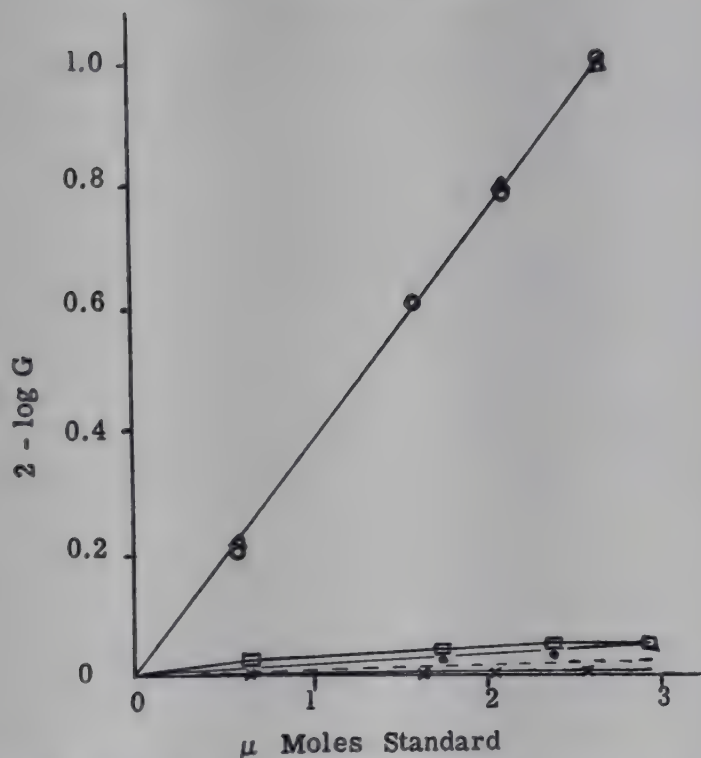


FIG. 1. Color development of a series of purines, allantoin, and uracil carried through the whole procedure. O, xanthine; Δ, guanine; □, hypoxanthine; ●, adenine; X, uracil and allantoin; dashed line, uric acid.

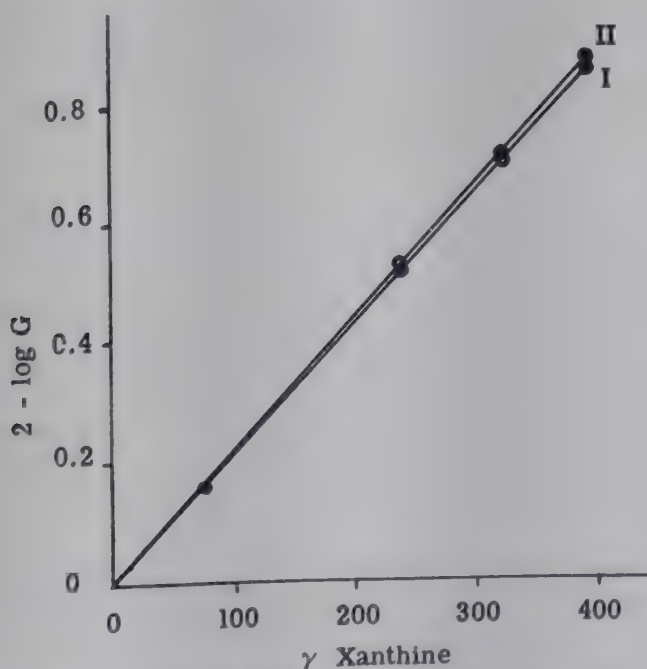


FIG. 2. Color development of a xanthine standard carried through the whole procedure in one case and only diluted in the other. Curve I, color development from the precipitated and recovered standards; Curve II, that from the diluted standard only.

ment on an equimolar basis. The wide range over which these two purines give a straight line relationship when concentration is plotted against $2 - \log$ (galvanometer reading) makes the method easily adaptable to visual types of colorimetry.

In order to check for complete precipitation and recovery of xanthine in the method, two series of xanthine standards were prepared. One series was precipitated and recovered according to the complete experimental procedure, and the other was only diluted with 5 N HCl, water, and ferri-cyanide according to the last part of the procedure. Results of these experiments are presented in Fig. 2. Curve I represents color development from the precipitated and recovered standards; Curve II, that from the diluted standard only. From these curves it is evident that precipitation

TABLE I

Recovery of Xanthine Added to Urine Obtained from Rat on Synthetic Ration

Urine volume	Xanthine added	Xanthine obtained	Recovery
ml.	γ	γ	per cent
2	0	5	
2	0	4	
2	240	220	90
2	400	370	93
4	0	8	
4	0	10	
4	240	235	94
4	400	380	93
Average.....			93

and recovery are practically 100 per cent when compared to the same unprecipitated standard.

The possibility that other substances besides xanthine and guanine are present in urine, which might be carried over and which might give positive color development, was checked by adding standard xanthine to a sample of urine obtained from rats which had been fed a synthetic ration (*i.e.*, casein, sucrose, fat, vitamins, and Salts IV (5)) for 2 weeks. Under these conditions the amounts of purines already in the urine should be negligible because of lack of exogenous purines. Results from these experiments are given in Table I. The standard xanthine was added to the fresh neutralized urine before the precipitation procedure was begun.

In order to obtain an average figure for the urinary xanthine and guanine excretion of rats fed an ordinary stock ration, eight animals weighing 80 to 90 gm. were placed in individual metabolism cages for 1 week. During this time they were fed a stock ration and given water *ad libitum*. Urine from

the animals was collected under toluene and at the end of the period analyzed by the foregoing procedure for xanthine and guanine. The results of these determinations, given in Table II, are reported as micrograms of xanthine and guanine excreted per rat per 24 hrs.

TABLE II
Urinary Excretion of Xanthine and Guanine, Reported As Xanthine, by Group of Male Rats Given Ordinary Stock Ration

Rat No.	Xanthine excreted per rat per day
	γ
1	118
2	121
3	272
4	157
5	75
6	171
7	330
8	179
Average.....	178

TABLE III

Xanthine and Guanine, Reported As Xanthine, in 12 Hour Urine Sample of Human Male on Normal Diet, with Recovery Values for Added Xanthine

Urine volume	Xanthine added	Xanthine obtained	Recovery
<i>ml.</i>	γ	γ	<i>per cent</i>
2	0	40	
2	0	40	
2	240	265	94
2	400	435	99
4	0	82	
4	0	90	
4	240	315	95
4	400	450	91
Average.....			95

A 12 hour urine sample from a human male on a normal diet was obtained and analyzed by this procedure for xanthine and guanine (reported as xanthine). Recovery values of added xanthine were also obtained in this case. These results are shown in Table III. Since the total volume of urine in the 12 hour sample was 650 ml., the 12 hour excretion of xanthine and guanine was calculated to be 14 mg.

DISCUSSION

It is quite possible that the precipitation procedure outlined in this report may be modified for determining total purine excretion, since all the purines tested are precipitated under the conditions given. By using larger volumes of urine, enough purine precipitate can be obtained for nitrogen determination. Also, application of spectrophotometric methods to the dissolved precipitate might give a means of determining adenine as outlined by Kerr *et al.* (6).

Clinical application of the method outlined in this report can be made quite easily because of the small volume of urine required in the analysis. It is also possible that measurement of excretion of xanthine and guanine may be applied diagnostically, since excretion of purine bases has been observed to be increased in certain disorders such as leucemia.

SUMMARY

A rapid and accurate method for measuring xanthine and guanine in small volumes of urine has been presented. Recovery values of xanthine added to rat and human urine varied from 90 to 100 per cent.

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PREPARATION AND ENZYMATIC HYDROLYSIS OF THREE NEW HOMOLOGOUS DEHYDROPEPTIDES

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The only dehydropeptides so far found susceptible to enzymatic hydrolysis are (a) the glycyl, sarcosyl, and L-alanyl derivatives of dehydroalanine and of dehydrophenylalanine (1, 2) and (b) the acetyl and chloroacetyl derivatives of dehydroalanine (1, 2). The former group of substrates is hydrolyzed by preparations of all the animal and plant tissues studied (dehydropeptidase I) (1). The latter group is hydrolyzed by only a few animal and plant tissues (dehydropeptidase II) (1-3). The acetyl and chloroacetyl derivatives of dehydrophenylalanine are completely resistant to the action of all the tissues studied (1, 2), as are the acetyl derivatives of dehydrotyrosine, dehydroaminobutyric acid, dehydrovaline, dehydroleucine, and dehydronorleucine (4). In view of the fact that the glycyl derivatives of dehydrophenylalanine are enzymatically susceptible, the question arises whether the glycyl derivatives of dehydroaminobutyric acid, etc., would also be enzymatically susceptible. Consequently, three new homologous dehydropeptides, glycyldehydroaminobutyric acid, glycyldehydronorvaline, and glycyldehydronorleucine, were prepared and incubated with preparations of various animal tissues. All three were found to be hydrolyzed by all the tissue preparations studied.

EXPERIMENTAL

Synthesis of Dehydropeptides—Price and Greenstein reported that chloroacetonitrile and α -chloropropionitrile condensed with freshly distilled and dry pyruvic acid in the presence of dry hydrogen chloride to form, respectively, chloroacetyldehydroalanine and α -chloropropionyldehydroalanine in good yield (1). The reaction proceeds smoothly and no formation of chloroacetamide has ever been noted.¹ Amination of the halogenated dehydropeptides in aqueous ammonia produced the corresponding glycyl- and DL-alanyldehydroalanines.

When this reaction was attempted with chloroacetonitrile and, respec-

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¹ For complete drying of the pyruvic acid, an azeotropic mixture of pyruvic acid with benzene has been generally employed.

tively, α -ketobutyric acid, α -keto-*n*-valeric acid, and α -keto-*n*-caproic acid,² in the presence of hydrogen chloride, condensation occurred to form the corresponding dehydropeptides. However, the products were generally admixed with 20 to 30 per cent of chloroacetamide and could not therefore be adequately characterized.³ This relatively large amount of impurity did not interfere with the preparation of the glycyldehydroamino acids, for on subsequent amination of the mixtures in concentrated aqueous ammonia the chloroacetamide was converted to glycineamide. Since glycineamide is completely soluble in alcohol, it could be readily separated from the dehydropeptide (5).

As before, 1 mole of chloroacetonitrile was mixed with 1.2 moles of either α -ketobutyric acid, α -keto-*n*-valeric acid, or α -keto-*n*-caproic acid (each redistilled four times *in vacuo*) and the mixture saturated at 5° with dry hydrogen chloride. After standing for 3 to 5 days at 5°, the mixture solidified into a yellow-brown crystalline mass. It was washed several times with petroleum ether, stirred with dry ether, and filtered. On solution in the minimum amount of warm acetone, followed by addition of petroleum ether, the products crystallized in the form of long, white needles. The crystallization procedure was repeated. Nitrogen and chlorine analyses on the dried products gave values considerably in excess of theory for the chloroacetylated dehydroamino acids and indicated the presence of appreciable amounts of chloroacetamide. Previous experience had shown that so much impurity could not be removed from the dehydropeptides by recrystallization. The products were dissolved in 20 times their weight of ammonia water which had been saturated at 0°, and the solutions were allowed to stand at 25° for 4 days. At the end of this period, the solutions were evaporated to dryness, and the residues washed several times with 95 per cent alcohol and filtered. The residues were then dissolved in the minimum amount of hot water, filtered, and treated with 3 times the volume of hot absolute alcohol. The glycyldehydroamino acids appeared on cooling as long, white needles. If any product was slightly colored, it was recrystallized in the same manner. The yield of each of the dehydropeptides was 20 to 30 per cent of the theory, based on the chloroacetonitrile taken. The compounds were free of ammonia and gave analytical values in close accord with the theory.

Glycyldehydroaminobutyric acid.	Calculated.	C 45.6, H 6.3, N 17.7
	Found.	" 45.2, " 6.3, " 17.6
Glycyldehydronorvaline.	Calculated.	C 48.8, H 7.0, N 16.3
	Found.	" 48.8, " 7.0, " 16.3

² We are indebted to Dr. Alton Meister for the preparation of α -keto-*n*-caproic acid.

³ The water involved in the formation of the amide may have been derived from some breakdown of the keto acids during the condensation.

Glycyldehydro-norleucine.	Calculated.	C 51.6, H 7.5, N 15.1
	Found.	" 51.6, " 7.5, " 15.0

Heated in capillary tubes, the compounds darkened above 200° and did not melt up to 230°.

Acid Hydrolysis of Dehydropeptides—Approximately 500 mg. of each of the dehydropeptides were dissolved in 15 cc. of 2 N HCl and refluxed for 1 hour. To the hydrolysate was added an equivalent amount of 2,4-dinitrophenylhydrazine, as a 1 per cent solution in hot 2 N HCl. The resulting precipitates were filtered, washed with cold water, and recrystallized from hot water. The yields of the 2,4-dinitrophenylhydrazones were 30 to 50 per cent, the low figures being due to some destruction of the keto acids during the acid hydrolysis. The compounds were compared with authentic samples of the 2,4-dinitrophenylhydrazones of α -ketobutyric, α -keto-*n*-valeric, and α -keto-*n*-caproic acids. The analytical data are as follows:

2,4-Dinitrophenylhydrazone of α -ketobutyric acid, m.p. 197–198°; N 19.6 per cent, N calculated 19.8 per cent. 2,4-Dinitrophenylhydrazone isolated from the hydrolysate of glycyldehydroaminobutyric acid, m.p. 197–198°; N 19.6 per cent. A mixture of the two products melted at 197–198°.

2,4-Dinitrophenylhydrazone of α -keto-*n*-valeric acid, m.p. 167°; N 18.9 per cent, N calculated 18.9 per cent. 2,4-Dinitrophenylhydrazone isolated from the hydrolysate of glycyldehydro-norvaline, m.p. 167°; N 18.9 per cent. Mixed m.p. 167°.

2,4-Dinitrophenylhydrazone of α -keto-*n*-caproic acid, m.p. 153°; N 18.1 per cent, N calculated 18.1 per cent. 2,4-Dinitrophenylhydrazone isolated from the hydrolysate of glycyldehydro-norleucine, m.p. 153°; N 18.1 per cent. Mixed m.p. 153°.

Ultraviolet Absorption Spectra—The peptides of dehydroalanine possess a characteristic absorption in the ultraviolet with a maximum at 2400 Å (1). The absorption spectra of the dehydrophenylalanine peptides, on the other hand, exhibit a maximum at 2750 Å (1, 6). A third type of absorption is exhibited by the peptides of dehydroaminobutyric acid, dehydro-norvaline, and dehydro-norleucine, for these compounds, although possessing considerable absorption in the ultraviolet, exhibit no characteristic maxima between 2200 and 2700 Å (Fig. 1). This finding was previously observed for the *N*-acetyl derivatives of several dehydroamino acids (4, 6). The marked difference in the character of the ultraviolet absorption of acetyldehydroalanine, on the one hand, and of acetyldehydroaminobutyric acid, on the other, was noted earlier (4), but an exact comparison was not feasible because the two types of compounds were synthesized by different procedures. However, in the case of the corre-

spending glycidylhydroxamine acids, both glycidylhydroxalanine and glycidylhydroxaminobutyric acid are synthesized by substantially the same procedure, and yet each in aqueous solution absorbs ultraviolet radiation differently. This curious difference between the absorption of glycidylhydroxalanine and that of its higher homologues is unexplainable at this time.

The absence of a characteristic maximum of absorption for the new hydropeptides precludes the use of the spectrophotometric procedure following enzymatic hydrolysis which had been applied to the cases of dehydroalanine (7, 8) and dehydrophenylalanine peptides (1, 6, 9).

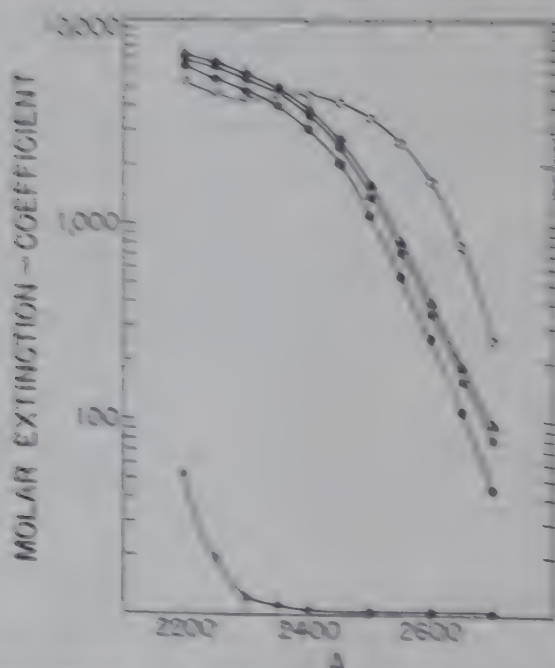


FIG. 1. Absorption curves in the ultraviolet of 1.7×10^{-4} aqueous solutions of various dehydropeptides. ● glycidylhydroxaminobutyric acid, ■ glycidylhydroxalanine, ▲ glycidylhydroxaminobutyric acid, □ glycidylhydroxalanine and ◆ glycidylhydroxaminobutyric acid are included for comparison.

Enzymatic Hydrolysis of Dehydropeptides—As previously noted, susceptible dehydropeptides are hydrolyzed by tissue preparation products which include ammonia and the corresponding α -keto acids in equivalent proportions (11). When the new substrates, glycidylhydroxaminobutyric acid, glycidylhydroxaminobutyric acid, and glycidylhydroxaminobutyric acid were incubated at 38° with fresh aqueous extracts of various rat tissues, it was noted that ammonia and the keto acids were readily produced in equivalent amounts. The keto acids, produced after complete hydrolysis of the respective substrates by extracts of rat kidney, were isolated from the deproteinized filtrates as the 2,4-dinitrophenylhydrazones. The yields were nearly theoretical. After recrystallization from water, 2,4-

hydrazones of the following keto acids gave the respective analytical data: α -ketoglutaric acid (m.p. 187-193°; N found 13.7, N calculated 13.6); α -ketonorvalic acid (m.p. 103°; N found 13.9, N calculated 13.9); ketonocaproic acid (m.p. 153°; N found 13.2, N calculated 13.1). Melting points with authentic samples of the 2,4-dinitrophenylhydrazones of the corresponding α -keto acids revealed no depression of the melting point in any case.

Initial rates of hydrolysis of the dehydropolypeptides were measured by appearance of ammonia in the digesta over that of the tissue controls.

The digesta generally consisted of 1 cc. of the fresh aqueous tissue extract, 2 cc. of 0.15 M borate buffer at pH 8.1, and either 1 cc. of water or 0.25 M substrate solution. Aqueous solutions of the substrates were generally stable and could be kept for several days in the ice chest without

TABLE I

Susceptibility of Dehydropolypeptides to Various Rat Tissue Extracts

Dehydropolypeptide	Micromoles $\pm$ 10 substrate hydrolyzed per hr. per mg. total N per cc. extract							
	Kidney	Liver	Pancreas	Brain	Spleen	Muscle	Testis	Lung
glycyldehydroalanine	1420	90	530	72	230	20	500	1180
glycyldehydroaminoacetic acid	900	20	140	5	10	4	50	140
glycyldehydronorvaline	540	20	135	5	10	4	50	130
glycyldehydronorleucine	420	15	130	5	10	1	50	130

of breakdown. The enzymatic data are given in Table I. For comparison, data on glycyldehydroalanine are included.

Though glycyldehydroalanine is readily hydrolyzed by all the tissues tested, extracts of rat brain, spleen, and muscle exhibit only minimum activity toward glycyldehydroaminoacetic acid, glycyldehydronorvaline, glycyldehydronorleucine. With other tissue preparations, the hydrolytic rates of the latter substrates are generally lower than that of glycyldehydroalanine.

The enzymatic susceptibility of glycyldehydroalanine in extracts of tumor is considerably higher than in corresponding normal tissues (12).

A comparison of the activity of mouse liver and of a transplanted hepatoma 13 in the same C strain of mouse revealed that, although susceptibility of glycyldehydroalanine was about 3 times greater in fractions of the tumor than in corresponding preparations of liver, activity toward glycyldehydronorvaline and of glycyldehydronorleucine was unaltered or even slightly less in the tumor preparation (Table II).

About 90 per cent of the activity toward glycyldehydroalanine in aqueous

extracts of rat kidney can be sedimented by centrifugation at 26,000 X for 2 hours (13). The same treatment also sediments the activity toward glycyldehydroaminobutyric acid, glycyldehydronorvaline, and glycyldehydronorleucine to approximately the same extent.

Effect of pH on Enzymatic Hydrolysis—The rate of hydrolysis of glycyldehydroaminobutyric acid, glycyldehydronorvaline, and glycyldehydronorleucine

TABLE II

Dehydropeptidase Activity in Mouse Liver and Transplanted Hepatoma 15

Dehydropeptide	Micromoles X 10 substrate hydrolysed per hr per mg. total N per cc. extract	
	Liver	Hepatoma
Glycyldehydroalanine	96	280
Glycyldehydronorvaline	13	15
Glycyldehydronorleucine	16	9

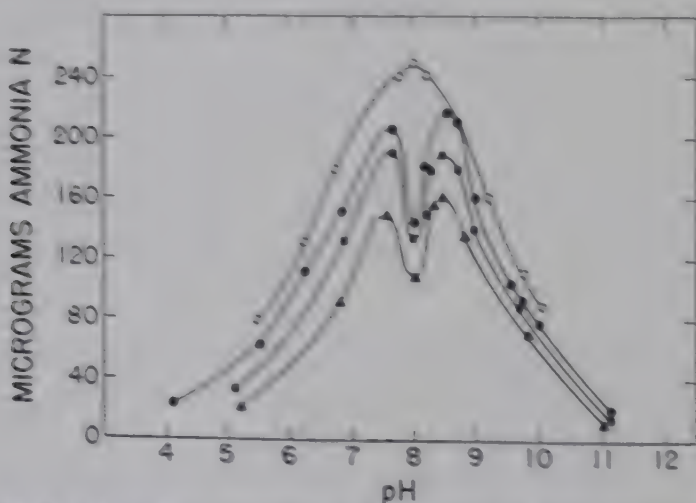


FIG. 2. Effect of pH on the enzymatic hydrolysis of dehydropeptides by aqueous extracts of rat kidney. 0.15 M borate buffers employed at pH 6.8 and above; veronal acetate buffers below pH 6.8. ● glycyldehydroaminobutyric acid, ■ glycyldehydronorvaline, ▲ glycyldehydronorleucine, ○ glycyldehydroalanine.

leucine by aqueous extracts of rat kidney bears an unusual relationship to the pH of the digest (Fig. 2). Two maxima of activity are observed: one at pH 7.6 to 7.7, the other at pH 8.5 to 8.6. There is a sharp minimum at pH 8.0. This type of pH-activity curve is unique among those of numerous dehydropeptides studied (2, 14). In contrast, glycyldehydroalanine exhibits a sharp maximum of hydrolysis at pH 8.0.

A study of the activity of rat liver extracts toward glycyldehydr

norvaline revealed a maximum hydrolysis at pH 8.6 only. This observation suggested the possibility that the two maxima shown by the kidney preparation might represent the presence of two enzymes, only one of which was present in liver. An attempt at separation was made by centrifuging an aqueous kidney extract at $26,000 \times g$ for 2 hours at 0° . The activity toward glycyldehydronorvaline which was concentrated in the sediment and that remaining in the supernatant exhibited the two original maxima, and the curves were practically superimposable.

The data in Table II and in Fig. 2 suggest that glycyldehydroalanine, on the one hand, and the three new dehydropeptides, on the other, are hydrolyzed by different enzymes. The possibility that dehydropeptidase I might be multiple in character has been expressed before (2). A fractionation of the separate activities in the particulate component of kidney would, however, be a difficult undertaking.

The authors are indebted to Mr. Robert J. Koegel for the elemental analyses.

SUMMARY

Glycyldehydroaminobutyric acid, glycyldehydronorvaline, and glycyldehydronorleucine have been prepared by condensation of chloroacetonitrile with the corresponding α -keto acid in the presence of hydrogen chloride, followed by amination of the condensation products in aqueous ammonia. These new dehydropeptides in aqueous solution exhibit a high but non-specific absorption of ultraviolet radiation.

On incubation with fresh aqueous extracts of several rat tissues, the dehydropeptides are hydrolyzed to products which include ammonia and the corresponding α -keto acid. With extracts of rat kidney, the susceptibility of the dehydropeptides shows two maxima, one at pH 7.6 to 7.7, the other at pH 8.5 to 8.6.

The distribution of enzymatic activity in various tissues toward the three new dehydropeptides is compared with that toward glycyldehydroalanine, and certain similarities and differences have been noted.

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METABOLISM OF FATTY ACIDS IN VITRO, STUDIED WITH ODD AND EVEN MEMBERS OF THE RC^{14}OOH SERIES*

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Studies concerning the metabolism of emulsified trilaurin ($-\text{C}^{14}\text{OO}-$) and octanoic acid ($-\text{C}^{14}\text{OOH}$) by rat tissue slices have been reported (1) in which the respired C^{14}O_2 was used as the criterion of metabolism. These experiments showed that liver possessed a lower capacity for the complete oxidation of fatty acids than did kidney, spleen, heart, and lung, especially at the lower substrate concentrations used. The most logical explanation for this difference between liver and the other tissues was a greater formation of acetoacetic acid by the former tissue, which would effectively remove substrate carbon from the major metabolic pathway involved in the complete oxidation of fatty acids.

The studies reported in the present paper involved a comparison of the ability of liver and kidney slices to form C^{14}O_2 and labeled acetoacetic acid from fatty acids. Carboxyl-labeled ($-\text{C}^{14}\text{OOH}$) pentanoic, hexanoic, heptanoic, octanoic, nonanoic, and dodecanoic acids were used as substrates to determine possible effects of the chain length and the odd or even character of the fatty acid. Only the carboxyl group of acetoacetic acid was assayed for radioactivity, although the carbonyl group has also been shown to be active (2-4).¹ Also included are studies on the effect of malonate on the incorporation of C^{14} into the carboxyl group of acetoacetic acid. Previous work (6) disclosed a reduction in C^{14}O_2 from fatty acids when malonate was present during the incubation with liver slices. Weinhouse *et al.* (2) have reported a similar observation, but, contrary to the results presented in this paper, acetoacetic acid formation was completely inhibited in their studies.

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¹ Experiments conducted in this laboratory have also disclosed that the carbonyl group is labeled. The ratio of its activity to that of the carboxyl group is not a constant, however, and, in accord with the findings of Crandall *et al.* (4, 5), is influenced by the chain length of the substrate. These experiments form the substance of another report.

EXPERIMENTAL

Carboxyl-labeled ($-\text{C}^{14}\text{OOH}$) pentanoic, hexanoic, heptanoic, and nonanoic acids were synthesized by the procedure previously used in the preparation of carboxyl-labeled octanoic and lauric acids (1). All of the acids were used as their sodium salts.

The incubations with liver or kidney slices from rats of the Wistar strain were performed either in the manner previously described (1) or by the usual Warburg technique; 125 ml. flasks equipped with two side arms and a center well were employed. At the end of the incubation period 1 ml. of H_3PO_4 was added and the flasks were shaken for 5 minutes. They were then chilled in an ice bath, and, when the Warburg flasks were used, an aliquot of the center well contents was removed for determinations of total carbon dioxide and activity (1). The tissue was removed from the flask and dried to constant weight, and the medium was diluted to 25 ml. with CO_2 -free water. A suitable aliquot was transferred to a large test-tube which contained 3 ml. of 50 per cent citric acid for every 10 ml. of medium used and 1 drop of caprylic alcohol. The tube was provided with a rubber stopper through which passed an inlet tube that extended to the bottom of the tube, and an outlet tube which was connected to an alkaline trap. Nitrogen or oxygen was passed through the tube for 10 minutes to remove traces of respired C^{14}O_2 . The outlet tube was then connected to an absorption tube containing 1 N CO_2 -free NaOH . 3 ml. of aniline-citrate reagent (7) were added rapidly to the contents of the tube, and, after being tightly stoppered, the tubes were placed in a water bath at 38° for 3 to 6 hours. During this period a slow stream of nitrogen or oxygen was passed through the contents of the tube to carry liberated C^{14}O_2 into the alkaline absorption column. The carbonate solution was then analyzed in the same manner as were the samples of respired C^{14}O_2 .

Experiments similar to those described above were performed with liver slices and various fatty acids, both in the presence and absence of malonic acid. The amounts of C^{14}O_2 and C^{14} -labeled acetoacetic acid formed were determined.

RESULTS AND DISCUSSION

Results of the experiments conducted with liver and kidney slices, in which no malonate was present, are given in Tables I and II. The previously reported difference between these two tissues with respect to their ability to form C^{14}O_2 from fatty acids is also apparent from the data given here. Kidney converted more of the labeled carboxyl group to carbon dioxide than did liver, whether the carbon chain of the substrate contained an even or odd number of carbons. Liver formed more C^{14}O_2 from the odd numbered acids relative to the acetoacetic acid than from the adjacent even

numbered ones, as is shown by the values for the ratio of $C^{14}O_2$ to acetoacetate ($C^{14}OOH$). With kidney slices, however, a consistently low ratio was observed with all acids. The results with liver are in accord with studies reported by Jowett and Quastel (8) in which non-labeled substrates were employed. These workers reported higher Q_{O_2} values for liver slices incubated with odd numbered acids than with even numbered ones, but the formation of acetoacetic acid was much higher from the latter acids than from the former. The magnitude of the differences in $C^{14}O_2$ formed from these two types of acids reported in the present paper is not as great

TABLE I

*Conversion of $RC^{14}OOH$ to $C^{14}O_2$ and $CH_3COCH_2C^{14}OOH$ by Liver Slices**

Experiment No.	Substrate (0.001 M)	Conversion to $C^{14}O_2$ †	Conversion to acetoacetate $C^{14}OOH$ †	A + B	$\frac{B}{A}$
		(A)	(B)		
140	Pentanoic	3760	5,240	9,000	1.39
	Hexanoic	2480	10,300	12,780	4.16
	Heptanoic	2920	6,000	8,920	2.06
	Octanoic	2090	9,350	11,440	4.47
	Nonanoic	1850	4,750	6,600	2.57
	Dodecanoic	1540	3,780	5,320	2.45
141	Pentanoic	4600	4,920	9,520	1.07
	Hexanoic	4320	10,900	15,220	2.52
	Heptanoic	5300	5,400	10,700	1.02
	Octanoic	4580	11,800	16,380	2.58
	Nonanoic	4460	3,380	7,840	0.76
	Dodecanoic	1390	1,800	3,190	1.29

* Each flask contained 20 ml. of calcium-free Ringer's phosphate solution at pH 7.0 and approximately 0.2 gm. of liver slices (dry weight). Incubation time, 30 minutes. The Warburg technique was used.

† Expressed as total counts per minute per gm. of dry tissue. All values have been corrected for differences in activities between the various substrates for convenience in making comparisons.

as the corresponding differences in Q_{O_2} values reported by Jowett and Quastel. The latter values, however, reflect the oxidation of other carbon atoms in the substrates and also of those in the liver slices themselves. Nevertheless, it would appear that the acids of an odd number of carbon atoms are preferentially oxidized rather than converted to acetoacetic acid, even in the case of liver slices. Evidence that the formation of acetoacetic acid from odd numbered acids is similar to that from acids of an even number will be presented in a subsequent paper.

The data in Table I show that the chain length of the fatty acid had little influence on the extent of complete oxidation of the radiocarbon.

Similar findings were reported by Weinhouse *et al.* (2) for carboxyl-labeled octanoic, hexanoic, butyric, and acetic acids. In the present paper no calculations have been made which are based on the assumption that all carbons of a given fatty acid have undergone the same degree of oxidation as the carboxyl group. Current studies are in progress with acids labeled in various positions to test the validity of such an assumption when dealing with tissue slices.

The inhibitory effect of malonic acid on the formation of $C^{14}O_2$ from $RC^{14}OOH$ by liver slices is shown by the data in Table III. This inhibition was obtained whether the acid was of an even or odd number of carbon

TABLE II
*Conversion of $RC^{14}OOH$ to $C^{14}O_2$ and $CH_3COCH_2C^{14}OOH$ by Kidney Slices**

Experiment No.	Substrate (0.001 M)	Conversion to $C^{14}O_2$ †	Conversion to acetoacetate $C^{14}OOH$ †	A + B	$\frac{B}{A}$
		(A)	(B)		
144	Pentanoic	7,650	2570	10,220	0.34
	Hexanoic	7,930	2880	10,810	0.36
	Heptanoic	8,400	2160	10,560	0.26
	Octanoic	8,100	3860	11,960	0.48
	Nonanoic	7,650	2270	9,920	0.30
	Dodecanoic	4,200	450	4,650	0.11
145	Pentanoic	10,800	2420	13,220	0.22
	Hexanoic	7,250	1300	8,550	0.18
	Heptanoic	7,520	790	8,310	0.11
	Octanoic	7,230	1770	9,000	0.25
	Nonanoic	9,250	1120	10,370	0.12
	Dodecanoic	4,150	310	4,460	0.07

* Conditions as given in foot-note of Table I. Approximately 0.06 gm. (dry weight) of kidney slices was used in Experiment 144 and 0.12 gm. of tissue (dry weight) in Experiment 145.

† Counts per minute per gm. of dry tissue.

atoms. Previous studies (6) with carboxyl-labeled octanoic acid and tri-laurin gave similar results, and it was further shown that various supplements, such as fumaric acid, did not counteract this inhibition. It was pointed out that there was no need to assume an effect of malonate upon the fatty acid oxidation itself. Instead, the simplest interpretation was to assume that blocking occurred only at the succinate-fumarate stage and prevented the liberation of much of the radiocarbon as $C^{14}O_2$. It was further assumed that the C^{14} would accumulate in succinic acid or other intermediates prior to the succinate-fumarate stage.

The data in Table III show that malonate did not appear to block fatty acid metabolism itself, for the total amount of C^{14} accounted for in the

$C^{14}O_2$ and the carboxyl group of acetoacetic acid in the presence of malonate was almost normal. The data suggest that the decrease in $C^{14}O_2$ which occurred when malonate was present was due to C^{14} being diverted into acetoacetic acid formation. How much radiocarbon was accumulated in such intermediates as succinic acid remains to be determined.

These data are contrary to those reported by Weinhouse *et al.* (2) which showed a decrease in $C^{14}O_2$ owing to malonate, but no formation of acetoacetic acid whatsoever in its presence. Our data are in agreement with those of Lehninger and Kennedy (9) who found almost quantitative conversion of fatty acids to acetoacetic acid when malonate was present. At

TABLE III

*Effect of Malonate on Conversion of $RC^{14}OOH$ to $C^{14}O_2$ and $CH_3COCH_2C^{14}OOH$ by Liver Slices**

Experiment No.†	Substrate (0.001 M)	Incubation time	Conversion to $C^{14}O_2$ ‡		Conversion to acetoacetate $C^{14}OOH$ ‡		A + B		$\frac{B}{A}$	
			(A)		(B)					
			Control	Malonate	Control	Malonate	Control	Malonate	Control	Malonate
		min.								
134	Pentanoic	90	14,100	6600	10,000	21,400	24,100	28,000	0.71	3.24
154	Hexanoic	60	4,600	1810	9,150	14,900	13,750	16,710	1.99	8.24
131	Heptanoic	90	10,900	3820	5,400	9,880	16,300	13,700	0.50	2.59
123	Octanoic	90	9,800	3910	11,200	15,100	21,000	19,010	1.14	3.86
134	Nonanoic	90	4,290	3080	7,350	14,200	11,640	17,280	1.72	4.61

* Conditions as given in foot-note of Table I.

† Experiment 154 performed by Warburg technique; all others by continuous O_2 flow technique (1).

‡ Counts per minute per gm. of dry tissue.

present, the only plausible reason for the difference between the results of Weinhouse *et al.* and those of Lehninger and ourselves is that of variation in different strains of animals, as suggested by Weinhouse *et al.* (2). The nature of the acetoacetic acid formed when malonate is present is currently being investigated.

SUMMARY

Studies on the metabolism of carboxyl-labeled pentanoic, hexanoic, heptanoic, octanoic, nonanoic, and dodecanoic acids, *in vitro*, are reported.

Liver slices converted more radiocarbon to acetoacetic acid ($-C^{14}OOH$) than did kidney slices, but the total C^{14} accounted for in this product and as respired $C^{14}O_2$ was similar for both tissues.

The amount of $C^{14}O_2$ produced from the various substrates by liver was

more uniform than was that of the acetoacetic acid ($\text{—C}^{14}\text{OOH}$) formed. The latter was higher for the even numbered acids than for the odd numbered ones.

Malonic acid decreased C^{14}O_2 formation from all substrates by liver slices, but simultaneously increased acetoacetate ($\text{—C}^{14}\text{OOH}$) formation.

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PHOSPHATASES OF LIVER

I. GLUCOSE-6-PHOSPHATASE*

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Shortly after the discovery of the process of phosphorolysis, it was suggested that the glucose of liver and blood is formed through the glucose phosphates (2). Several investigators (3-8) have studied the reactions in liver extracts in order to determine the nature of the phosphatase which split the glucose phosphate. The weight of the evidence favors the concept that neither G-1-P¹ nor F-6-P is hydrolyzed, but both are first converted to G-6-P, and this is the only hexose monophosphate which is split. Fantl and Rome (7) incubated G-1-P, Embden's ester, and F-6-P with crude and dialyzed liver extracts and compared the amounts of free glucose and fructose formed with the changes which took place in the inorganic and acid-labile phosphate. From these data, and also from some inactivation experiments, they concluded that the phosphatase is specific for G-6-P, and is distinct from the enzyme which split Gly-P at pH 6 to 7 in their experiments. Broh-Kahn and Mirsky (8) used G-1-P as substrate, and compared the rate of disappearance of acid-labile P to the rate of appearance of inorganic P. They found that G-1-P disappeared more rapidly than inorganic P appeared, and that whatever inhibited the former reaction also inhibited the latter.

However, all these authors have used crude liver extracts with high activities of other enzymes; hence their evidence is all indirect. Indeed the possibility remains that, in an extract in which phosphoglucomutase activity is higher than that of the phosphatase, the G-1-P may be so rapidly converted that it could not be split, even if there were a G-1-Pase. Because of the intrinsic importance of these reactions for the mechanism of production of blood sugar, it seemed worth while to attempt purification of the G-6-Pase and to study its properties in purified form. It was found possible to prepare a fraction with high activity toward G-6-P, no activity

* A preliminary report was presented before the meeting of the American Society of Biological Chemists at Detroit, April, 1949 (1).

¹ In order to conserve space, the following abbreviations will be used: G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; HDP, hexose diphosphate (fructose-1,6-diphosphate); Gly-P, glycerol phosphate; ATP, adenosine triphosphate; TCA, trichloroacetic acid.

toward G-1-P, and reduced activity toward other phosphate esters. The effects of pH and of various salts on the activity of the purified preparation and the distribution of the enzyme among certain cellular substructures were also studied. After the experimental work had been completed and the results summarized in a preliminary report (1), there appeared a paper by de Duve *et al.* (9) in which purification of the enzyme was also described. Several of his observations are in agreement with those reported in this paper.

EXPERIMENTAL

Preparation of Substrates

Glucose-1-phosphate was prepared by either the method of Sumner and Somers (10) or that of McCready and Hassid (11). Both materials contained the theoretical amount of easily hydrolyzable phosphorus and no inorganic or acid-stable phosphorus.

Fructose-6-phosphate was obtained as the barium salt after the hydrolysis of HDP in HBr, as described by Neuberg *et al.* (12).

Fructose-1,6-diphosphate was obtained from the Schwarz Laboratories, Inc. As supplied, it contains a considerable amount of free phosphate, which was eliminated by passage through the acid barium salt of the HDP, according to Neuberg (12).

Glycerol phosphate, sodium salt, was used as obtained from Merck and Company, Inc. It contained the theoretical amount of organic phosphate, and no inorganic phosphate. From the periodate titration, it was calculated to consist of 66 per cent α -glycerol phosphate. The β ester, which was not available until the experiments were nearly completed, was obtained from the Bios Laboratories, Inc., as the sodium salt.

Glucose-6-phosphate—Three samples were used. One, prepared synthetically, was the gift of Dr. Bernard Horecker. According to his statement, it was 92 per cent pure by enzyme assay. Another sample was prepared by the method of Colowick and Sutherland (13). Since the yield by their method is very low compared to the amount of time and labor required, a simpler procedure was devised, similar to that used for preparation of G-1-P but with the addition of muscle extract to furnish phosphoglucomutase activity from the beginning. In this system, 95 per cent of the esterified phosphate is converted to the 6-ester and, therefore, no large excess of phosphate is required. Since the details were combined from several published methods, an outline of the preparation will be given.

Finely ground muscle was extracted twice with 2 volumes of cold water. Then 0.1 volume of 1 M acetate buffer, pH 5.2, was added, and the temperature brought quickly to 60° and held for 3 minutes. The mixture

was then cooled in ice to 4°, filtered, the filtrate brought to pH 7.4, and filtered again.² Potato extract was prepared by blending 150 gm. of white potatoes in 100 ml. of water, straining to remove coarse particles, and allowing the starch to settle somewhat. A solution of soluble starch, 9 gm. (0.05 mole) and 0.05 mole of sodium phosphate, pH 6.8, in about 600 ml. of water was allowed to cool to room temperature, and then 100 ml. of the potato extract and 100 ml. of heated muscle extract were added. The mixture was covered with toluene and allowed to stand at room temperature. When 50 per cent of the phosphate was esterified (about 24 hours were required), the reaction was considered complete. The solution was deproteinized by heat, the phosphate removed with Mg^{++} , and the esters purified over exchange resins essentially as described by McCready and Hassid (11). After elution, the barium salts of the esters were precipitated by ethanol, G-1-P hydrolyzed in 1 N HCl, and barium phosphate removed. The barium salt of G-6-P was purified in the usual way by repeated precipitation from water with ethanol. From 9 gm. of starch about 5 gm. of the anhydrous barium salt, 95 per cent pure as calculated from its phosphorus content, were obtained.

Preparation of Liver Extracts

Albino rats, not fasted, were killed by decapitation. The livers were removed as rapidly as possible, ground in cold solution in a Waring blender or in a Potter homogenizer, and centrifuged in the cold. In various experiments, distilled water, 0.9 per cent NaCl, 1 per cent glucose, 0.1 M citrate at pH 6.5, and 30 per cent sucrose were used as extraction media. The extracts were routinely tested against G-6-P as described below. The fineness of grinding and the kind of solution used influenced the amount of protein extracted and also the enzymatic activity per mg. of protein, but these points were not investigated systematically. It was found that the major part of the activity was lost by even a short exposure to room temperature, or by storage in a refrigerator at 3° for a few hours. The presence of glucose protected somewhat against this inactivation. The small residual activity was resistant even to incubation at 40° for several hours. The original activity of the extracts could be maintained for several months by storage in a freezing cabinet at -30°. The enzyme is sensitive to low pH, and this, combined with its lability to temperatures above the freezing point, may account for the losses occurring during dialysis. This point is of some interest, especially when contrasted with the stability of other phosphatases.

² A considerable precipitate forms at about pH 6.5 to 6.8. If the precipitate is removed, the solution contains no mutase activity. By bringing the pH to 7.4, all but a small amount of this precipitate redissolves.

Test System

The test system which was adopted consisted of 0.1 ml. of substrate previously brought to pH 6.5, 0.2 ml. of buffer (usually 0.1 M citrate, pH 6.5), and 0.1 ml. of enzyme added last. For convenience, when the substrate had been prepared as the barium salt, it was converted to the sodium salt and brought to pH 6.5. In some early experiments, the barium salt was used directly, with no apparent effect on the enzymatic activity. At the end of the incubation period (30 minutes at 30° unless otherwise noted), 1 ml. of 10 per cent TCA was added. After a few minutes in ice, the samples were diluted to 2.5 ml. and centrifuged. A 1 ml. aliquot was used for determination of the phosphate by the method of Fiske and Subbarow (14), adapted for use with a Klett-Summerson photoelectric colorimeter. Appropriate tissue and substrate blanks were included in each series. The corrections due to these blanks were very small compared to the amount of phosphate liberated. When a substance was to be tested for activating or inhibitory effects, 0.1 ml. of a solution of that substance in buffer was substituted for a like amount of the buffer.

The enzyme solutions were diluted so that 60 to 75 γ of P were liberated from G-6-P in 30 minutes. This corresponded to about 20 to 25 per cent hydrolysis of the substrate. Activity was proportional to time up to 180 minutes, even if the substrate was 70 per cent hydrolyzed in the interval. Dilution of the extracts did not cause a proportional decrease in the activity; however, this caused no serious difficulty, since comparisons were almost always made between equal portions of the same solutions.

Purification and Specificity of Enzyme

In some preliminary experiments, the properties of the enzyme as a protein were investigated. It was found that the activity was not adsorbed on aluminum hydroxide, was precipitated at pH 5.5 (hereafter referred to as "isoelectric precipitation"), and was precipitated by half saturation with ammonium sulfate at 0° and pH 7.7. Even brief contact with acetone or ethanol at 0° and in concentrations as low as 10 per cent completely and irreversibly destroyed the enzymatic activity.

In Table I are given the results of one experiment in which the activity toward various substrates was tested at successive stages in an attempt at purification. It can be seen that the combination of isoelectric precipitation and treatment with aluminum hydroxide raises the activity toward G-6-P about 3-fold. The decrease in activity in the fraction precipitated with ammonium sulfate (and subsequently dialyzed to remove the excess salts) is possibly due to inactivation during dialysis, since in this last step the reaction became acid and a precipitate of apparently denatured protein formed.

The slight activity toward G-1-P which occurred in the crude dialyzed extract was entirely eliminated by the subsequent treatment. When Mg^{++} ions (which are necessary for some phosphatases and for mutase activity) were added to the ammonium sulfate precipitate, the ability to split G-1-P was not restored. In these digests, 100 per cent of the acid-labile phosphate was recovered after the incubation period. Therefore, it appears that the enzyme hydrolyzing G-6-P does not split G-1-P, and can be completely freed of phosphoglucomutase.

HDP was not split by the particular extract used in the experiments reported in Table I, although some other extracts did have some slight activity. This was perhaps to be expected, since Gomori (15) had shown that liver hexosediphosphatase has little activity at the pH used in these tests.

TABLE I

Activity of Rat Liver Phosphatase toward Various Substrates at Successive Stages in Purification

Values given as micrograms of P liberated per mg. of protein in 30 minutes at 30°. Substrate concentration 0.025 M, pH 6.5, 0.1 M citrate buffer.

Substrate	Whole dialyzed extract	Isoelectric ppt.	Aluminum hydroxide supernatant	Ammonium sulfate ppt.
Glucose-6-phosphate.....	24.9	56.0	72.5	51.5
Glucose-1-phosphate.....	7.9	4.9	2.0	0
Fructose-6-phosphate.....		46.1	45.5	25.1
Hexose diphosphate.....	0	0		
Glycerol phosphate.....		12.9	6.6	7.5

The rate of splitting F-6-P, in relation to the activity toward G-6-P, is significantly decreased during the purification of the extracts. Certain other experiments should perhaps be quoted in this regard. Since it has been shown (2, 7) that only glucose was liberated from Embden's ester and F-6-P by liver extracts, it was thought that hexoseisomerase was necessary to convert F-6-P to G-6-P before hydrolysis. Therefore, in two experiments in which F-6-P was used as substrate with extracts at various stages of purification, the amount of fructose was determined, as well as the amount of free phosphate, before and after incubation. In both experiments, the decrease in the rate of disappearance of fructose was the same as the decrease in the rate of appearance of inorganic phosphate. This is additional evidence that F-6-P is not split directly, but only after conversion to G-6-P.

The activity toward Gly-P became somewhat lower as compared to the activity against G-6-P, but never completely disappeared in the fraction-

ation procedures illustrated in Table I. In many other experiments, the activity toward both substrates was studied under various conditions, but the ratio of the rates observed varied only from 3 up to 6.5. A number of attempts to demonstrate competition between the two substrates have so far led to inconclusive results. Further work has been planned to study the question of whether or not these two substrates are split by the same enzyme.

On the basis of the experiments reported in Table I, the following procedure was adopted to prepare the enzyme for further study: An extract was prepared in 1 per cent glucose solution (4 ml. per gm. of liver), and the coarse particles removed. Then 0.5 volume of aluminum hydroxide was added, and the precipitate immediately centrifuged and discarded. The volume of the supernatant was measured, an equal volume of 0.1 M acetate of pH 5.2 was added, and the precipitate centrifuged immediately. This precipitate was taken up in a few drops of 2 per cent sodium carbonate and then diluted appropriately with 0.1 M citrate (or sometimes 0.1 M maleate) buffer at pH 6.5. All solutions were kept in ice and all centrifugations were carried out in the cold.

Effect of pH

The substrate solutions (0.1 ml.) were mixed with a sufficient quantity of 0.1 M solutions of acetate, citrate, or bicarbonate buffer to give 0.9 ml. samples of differing pH. Then 0.1 ml. of enzymes was added to each tube, and all samples were incubated at 30° for 30 minutes. At the end of this time, 0.5 ml. aliquots were removed, added to TCA, and reserved for the P determinations. To the remaining portion of the digest, 0.5 ml. of water was added and the pH determined with a small glass electrode. The results are plotted in Fig. 1. With G-6-P, the maximum splitting occurs at about pH 6.5. The general shape of the curve resembles that reported by Fantl and Rome (7) for crude extracts acting on Embden's ester, although in the present experiments the peak is somewhat sharper.³ When F-6-P is the substrate, the optimum pH is at a more alkaline point. This could mean either that there is a second phosphatase specific for F-6-P, with a higher optimum pH, or that the activity of hexoseisomerase, which presumably has a higher optimum pH, is the limiting factor. The latter explanation is the more probable in view of the fact that the total amount of fructose decreases as phosphate is liberated in this system, which would not be the case if F-6-P were split directly.

When Gly-P is used, the amount of phosphate liberated is small, but

³ It happens that pH 6.5 is also the optimum found by Fantl, Rome, and Nelson (5) for the liberation of glucose from starch. Taken at its face value, this coincidence might mean that the hydrolysis of the ester was the rate-limiting step in their system.

there does seem to be a maximum in the curve between pH 6 and 7, which agrees with the findings of Fantl and Rome. Woodward (16) reported a minimum at pH 6.0, after an autolysis which should certainly destroy the G-6-Pase. Although Fantl and Rome call this enzyme which splits Gly-P "acid phosphatase," it appears that the splitting is not due to the groups of enzymes usually known as "acid" or "alkaline" phosphatases.

Effect of Activators and Inhibitors

Neither Ca^{++} nor Mg^{++} affects the rate of splitting any of the five substrates. These ions were used in concentrations up to 0.05 M, and always

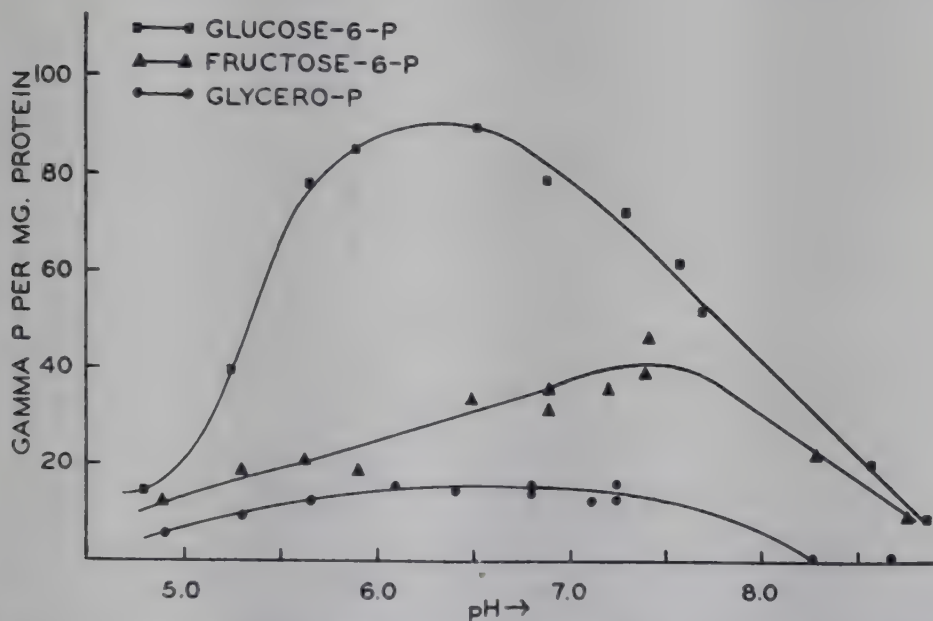


FIG. 1. Influence of pH on the activity of partially purified rat liver extract on glucose-6-phosphate.

at pH 6.5, in both maleate and citrate buffers. Histidine, arsenite, iodoacetate, and cyanide had no effect up to 0.05 M and at pH 6.5. Phlorhizin, up to 0.5 per cent, did not affect the rate of splitting either G-6-P or Gly-P. This is in contrast to most results previously reported (see especially (7, 8)). Fluoride, arsenate, and molybdate, on the other hand, are inhibitory. The effect of these three substances was studied in more detail, with both G-6-P and Gly-P as substrates. In order to obtain analytical values of sufficient magnitude to be significant, the concentration of the Gly-P was increased 5-fold, to 0.125 M. In the experiments with added arsenate it was necessary to remove the arsenate before the determination of inorganic P was made, since in this determination arsenomolybdic acid is reduced to a blue color at an unpredictable rate. For this purpose, the method of Pett (17), in which arsenate is converted to arsenite by sulfurous

acid, was found satisfactory. With this additional treatment, concentrations of arsenate corresponding to as high as 0.1 M in the enzyme digest were added to the standard, and the correct reading was obtained. The conditions of heating (30 minutes at 50°, in 0.5 N sulfuric acid) do not hydrolyze either G-6-P or Gly-P.

The degrees of inhibition caused by various concentrations of fluoride, arsenate, and molybdate are plotted in Figs. 2 and 3. It can be seen that the action of fluoride is rather erratic. It appeared that the more active extracts were more easily inhibited, and that the degree of inhibition depended in some degree on the treatment which the preparation had received prior to testing. These observations suggest that some of the dis-

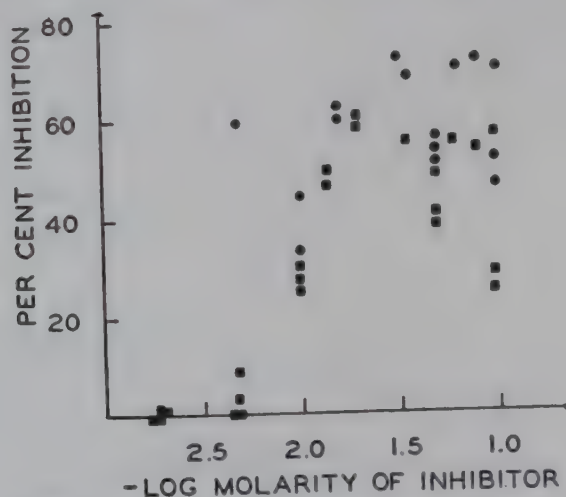


FIG. 2. Inhibition by fluoride of phosphatase activity in partially purified rat liver extracts. Per cent inhibition, $(\text{control} - \text{inhibited}) \times 100 / (\text{control})$. ●, glycerol phosphate 0.125 M as substrate; ■, glucose-6-phosphate 0.025 M as substrate, at 37°, 30 minutes; pH 6.5, 0.1 M citrate buffer.

crepancies recorded in the literature regarding the effect of fluoride on the formation of glucose in liver extracts may be due to unnoticed and unreported differences in the preparation of the extracts. The interference of arsenate with the methods for determining phosphorus makes it rather cumbersome to use in any system requiring such determinations. On the other hand, the use of molybdate does not present any difficulties.

Localization of Enzymes within Cell

Preparations of the large granules (mitochondria) from sucrose homogenates of rat liver have been made by the technique of Hogeboom, Schneider, and Pallade (18), as modified by Kennedy and Lehninger (19). It was found that the granules retained a significantly high activity toward G-6-P, which could not be altered by repeated washing. In order

to study the distribution of the enzyme in the various fractions, the following experiment was performed. From one liver, a small piece was taken and homogenized in water. The major portion was homogenized in 30 per cent sucrose and fractionated by centrifugation. The sediment of the cells and nuclei and that of the large granules were each washed four times with 0.15 M NaCl. The washings were combined with the unfractionated supernatant solution, and made up to a definite volume. Samples of the water and of the whole sucrose homogenates were diluted in the same proportion as the supernatant. The sediments of the nuclei and of the large granules were suspended in 0.15 M NaCl and made up to a volume

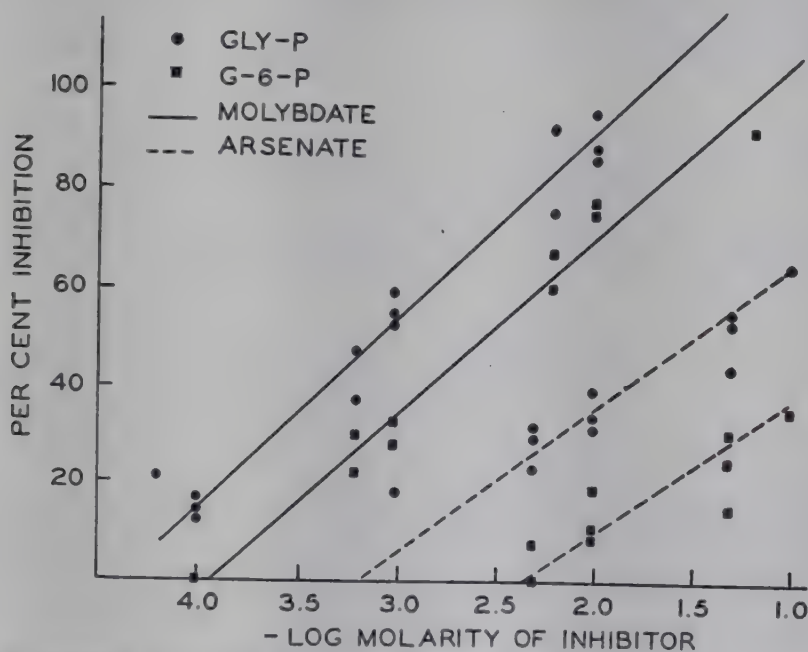


FIG. 3. Inhibition by molybdate and arsenate of phosphatase activity in partially purified rat liver extracts. Other conditions as for Fig. 2.

equal to twice the weight of the original tissue. Each of these fractions was tested against several substrates. The water homogenate was included in the series to determine whether sucrose inhibited the enzymes, since sucrose was present to a final concentration of 3 per cent in the digests containing the whole homogenate and the supernatant, while it was absent from the suspensions of the sediments.

It can be seen from Table II that, while there are some differences between the water and sucrose homogenates, sucrose does not seem to be inhibitory at this concentration. It can also be seen that most of the original phosphatase activity remains in the unfractionated portion containing the soluble proteins (and submicroscopic granules). If it is accepted that the phosphatase activity is specific for G-6-P, and that the

activity toward G-1-P and F-6-P is dependent on the presence of mutase and isomerase respectively, then the values in Table II indicate that the latter enzymes are also present in the soluble protein. The granules were tested in order to discover whether they might be a satisfactory preparation in which to study some of the reactions in the glycolytic cycle, such as the direct phosphorylation of glucose, which have been difficult to demonstrate because of the phosphatase activity. However, the activity of G-6-Pase, either integral in, or firmly adherent to, the granules, is sufficiently high to interfere seriously with such efforts, unless a satisfactory inhibitor can be found. Molybdate seems promising for this purpose. It remains to be seen whether molybdate interferes with enzymes acting on phosphorylated intermediates.

TABLE II

Distribution of Glucose-6-phosphatase among Fractions Separable from Liver Homogenate by Centrifugation

Substrate concentration 0.025 M, pH 6.5. Values expressed as micrograms of P liberated per 100 mg. of original tissue in 30 minutes at 37°.

Substrate	Water homogenate	Sucrose homogenate			
		Whole homogenate	Cells and nuclei	Large granules	Unfractionated supernatant
Glucose-6-phosphate.....	911	900	26	158	630
Glucose-1-phosphate	70	220	0	9	75
Fructose-6-phosphate	655	780	16	45	483
Hexose diphosphate.....	118	62	0	4	0
Glycerol phosphate.....	238	198	4	24	155

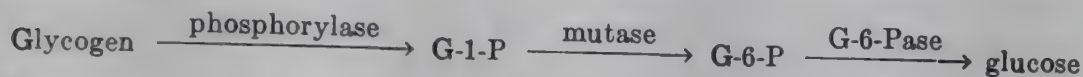
Both β -Gly-P and the α - β mixture were tested in these experiments with the homogenates and their fractions. There was no difference between the rate of splitting the β form and the rate of splitting that sample in which the Gly-P was predominantly in the α form.

DISCUSSION

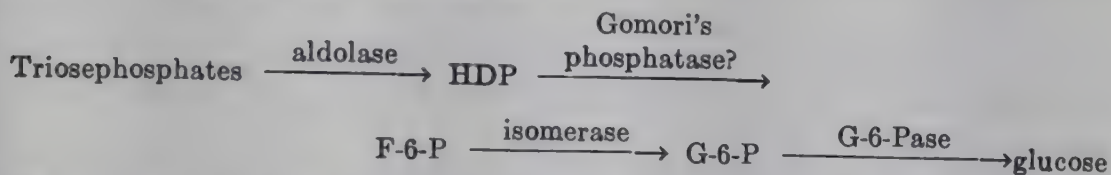
The results reported in this paper lead to the same general conclusion reached by other authors; namely, that there is in rat livers an enzyme which can catalyze the splitting of G-6-P but not of G-1-P and F-6-P. From the degree of inactivation which occurred in the present experiments with a 5 minute centrifugation at summer temperature, it seems likely that the other authors worked with enzyme preparations already 80 to 90 per cent inactivated. This may account for the fact that in the experiments of other workers all three hexose phosphates were split at nearly the same

rate, while we found G-6-P always to be split most rapidly. Fantl and Rome state that the phosphatase could be inactivated while the mutase and isomerase were still active. Our purification process leads to results which are the converse of theirs, in that the phosphatase was still highly active but had been completely freed of mutase activity and partially freed of isomerase.⁴ The fact that whole homogenates hydrolyze G-6-P so much more rapidly than G-1-P suggests that a specific G-1-Pase is not present in the liver.

It is recognized that there is always some question about the value of direct extension of results obtained from tissue extracts to the metabolism of a substance in an intact cell. With this reservation in mind, it seems that the evidence now available on the formation of blood sugar from liver glycogen can be most satisfactorily included in the following series of reactions.



The production of glucose from non-carbohydrate sources should include the following reactions as final steps.



By analogy with the latter series, one might also expect that, whatever galactose ester may be formed from dietary lactose, the ester must also be transformed to G-6-P before being split. Therefore, if it were demonstrated that liver tissue could liberate phosphate from a galactose phosphate, this might be taken as presumptive evidence that the galactose-transforming enzyme system was present.

As we might conceive the economy of the cell, it seems advantageous to have only one splitting enzyme like G-6-Pase so that mechanisms regulating the rate of reaction may be simpler and more effective. At the present time it is difficult to imagine what sort of control mechanism may be present. The great activity of the G-6-Pase (as well as that of the ATPase) in isolated liver tissue has prevented the demonstration of reactions such

⁴ At the beginning of this study, it was hoped that, if a material could be prepared which specifically split G-6-P and not any other phosphate esters, this material could be used advantageously for the separation of G-6-P from other esters. Such a separation represents a very difficult problem, the solution to which is badly needed, especially for experiments in which P^{32} is used as a tracer. Unfortunately, since the enzyme is very labile, a high degree of purity was not attained, and the level of purification achieved is not sufficient to allow the use of our material as an analytical tool.

as the phosphorylation of glucose. Yet, if our current concepts are at all correct, this reaction must take place with great readiness in the intact tissue.

SUMMARY

1. The enzyme in liver tissue which hydrolyzes glucose-6-phosphate has been partially purified. The purified preparation is free of phosphoglucose mutase activity and does not split glucose-1-phosphate at all. The diminution in the rate of splitting fructose-6-phosphate parallels the decrease in the hexoseisomerase activity.

2. The glucose-6-phosphatase was found to be extremely labile to heat, and most of the activity is lost by even a short period at room temperature. Its optimum pH is at a point where the action of phosphatases is usually found at a minimum. These facts together are taken as evidence that glucose-6-phosphatase is not part of the systems commonly designated as "acid" or "alkaline" phosphatases.

3. Glucose-6-phosphatase is neither stimulated nor inhibited by calcium and magnesium ions. It is not affected by arsenite, iodoacetate, cyanide, or histidine, up to 0.05 M, nor by phlorhizin up to 0.5 per cent. It is strongly inhibited by molybdate, less strongly by arsenate and fluoride. The inhibition by fluoride is erratic and appears to depend in part on the past history of the extract.

4. The enzyme occurs both in the soluble protein and in the large granules of the cell. Repeated washing of the granules did not remove the enzymatic activity.

5. All fractions which were active in splitting glucose-6-phosphate were also slightly active toward glycerol phosphate. The question is left open whether the two activities are due to one or two proteins.

The author wishes to thank Dr. Camillo Artom for his constant interest and helpful criticism of this work.

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OXIDATION AND TRANSAMINATION OF GLUTAMATE BY TYPHUS RICKETTSIAE*

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It has been previously reported (1) that partially purified rickettsiae are capable of oxidizing glutamate and that the rate of this reaction is proportional to the number of viable organisms present. It seemed of considerable interest to extend these observations in an effort to obtain more detailed information concerning the nature of the reactions involved, and to search for other reactions which these obligate intracellular organisms might catalyze. Although this work has been somewhat hampered by the difficulty in obtaining sufficient quantities of rickettsiae for analytical work, it has been possible to determine some of the end-products of glutamate oxidation and to establish the occurrence of a rickettsial transaminase.

Methods

Solutions—For the sake of brevity, the composition of the solutions used in preparation of the rickettsiae is listed below and these will subsequently be referred to by the designated abbreviation.

K-7G—KCl, 0.118 M; NaCl, 0.0072 M; Na_2HPO_4 , 0.0076 M; KH_2PO_4 , 0.0040 M; potassium glutamate, 0.0049 M; pH 7.0.

K-7.5—KCl, 0.126 M; NaCl, 0.0018 M; Na_2HPO_4 , 0.0106 M; KH_2PO_4 , 0.0012 M; pH 7.6.

KN—KCl, 0.122 M; NaCl, 0.023 M.

KN-G—100 ml. solution of KN + 3.0 ml. of 0.168 M potassium glutamate, pH 7.0.

Sucrose-P—Sucrose, 0.225 M; KH_2PO_4 , 0.0016 M; K_2HPO_4 , 0.0086 M, pH 7.5.

Tris-7.5—100 ml. of 0.3 M trishydroxymethylaminomethane + 75 ml. 0.3 M HCl, pH 7.5.

Sucrose-tris—90 ml. of 0.25 M sucrose + 10 ml. of tris-7.5.

6 per cent albumin—A solution of either bovine or human serum albumin. The bovine serum albumin, Armour's Fraction V, from bovine

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serum, was dissolved in salt KN. The human serum albumin¹ was obtained from the Massachusetts Department of Health, Division of Biologic Laboratories, as a 25 per cent solution, containing 1 per cent sodium acetyl-DL-tryptophan. It was dialyzed for 2 days against solution KN. Both albumin solutions were adjusted to the desired pH with 0.2 N KOH, diluted with solution KN to a concentration of 6 gm. per 100 ml., and sterilized by filtration through a Seitz filter.

Preparation of Rickettsiae—The rickettsiae were prepared from frozen infected yolk sac pools by the method previously described (1) with a few modifications. Sucrose, 0.05 M, was included in the salt solution used in homogenizing the yolk sacs because of the protective effect of this substance against loss of viability on freezing (2). Solution K-7G was used as diluent and suspending fluid for purification of the infected yolk sac. The precipitate from the final high speed centrifugation was resuspended in salt K-7.5 if it was to be used at once. If it was to be stored at -72° , as was frequently necessary, it was resuspended either in 6 per cent albumin, pH 7.4, or in sucrose-P. Control experiments showed that the albumin had no effect on the rate of O_2 uptake with glutamate whereas, sucrose, at the final concentration used, reduced the rate by about 20 per cent.

For certain experiments in which phosphate requirements were to be studied, phosphate was omitted from the solutions used in the above procedure. Solution KN-G was used for washing the rickettsiae in place of K-7G and sucrose-tris for resuspension of the final precipitate. The "tris" buffer had no effect on the rate of oxygen uptake.

It was necessary in some of the later work to use more highly purified rickettsiae than were obtained by this method. A mere repetition of the cycle of low and high speed centrifugation with the same diluent led either to large losses in activity or to little further purification. It was, however, noted that when the rickettsial preparations were suspended in 6 per cent albumin, a precipitate containing the major portion of the yolk sac material separated fairly rapidly. Therefore, the final precipitate of rickettsiae obtained in the usual method was resuspended in 6 per cent bovine albumin or human albumin, pH 7.0, with 1 ml. of albumin solution per gm. of original yolk sac. This suspension was centrifuged for 15 minutes at 800 r.p.m. The supernatant was carefully removed from the loosely coagulated tissue particles, diluted with 3 volumes of solution K-7G, and centrifuged at 5000 r.p.m. in an angle centrifuge for 45 minutes. The precipitate was resuspended to give a final volume of 0.5 ml. per gm. of original yolk sac either in salt K-7.5 or in sucrose-P. In a few experiments the phosphate-free solutions KN-G and sucrose-tris were substi-

¹ This was obtained through the courtesy of Dr. Dwight Mulford.

tuted for K-7G and sucrose-P respectively. The yield in this second purification cycle was 70 to 75 per cent. Normal yolk sac treated in this way gave only a trace of material in the fraction corresponding to the rickettsial precipitate, whereas after treatment only by the initial procedure, the corresponding fractions from normal pools were frequently indistinguishable macroscopically from those obtained from infected pools.

Two strains of rickettsiae have been used, the Breinl strain of *Rickettsia prowazeki* and the Wilmington strain of *Rickettsia mooseri*. More experiments have been done with the latter, as this strain yields more regularly yolk sacs sufficiently rich in rickettsiae for metabolic studies.

Oxygen consumption was measured by the usual Warburg method at 34.3°. CO₂ production was measured by the direct three flask method (3) in which one reaction flask contains alkali in the center well and a second does not. It was found that both glutamate utilization and ammonia production were about 10 per cent higher in the flask that contained KOH than in the one that did not; thus the manometric readings could not be used directly for estimation of CO₂ formation. Since NH₃ formation bore the same ratio to glutamate disappearance in the alkali and alkali-free flasks, it seemed probable that the same reactions were taking place in both and that only the rate differed. Therefore, since the difference in any case was small, it seemed that no great error would be involved in assuming for calculation of CO₂ formation that the oxygen consumption in the alkali-free flask was lowered proportionately to the decrease in NH₃ production, this being the more accurate analysis, and conversely that CO₂ production had been higher in the same proportion in the flask containing alkali. However, this assumption may not be correct and therefore the CO₂ values must be considered only approximate.

Unless otherwise specified, the reaction flasks contained 1.2 ml. of rickettsial suspension, sufficient 0.1 M potassium phosphate buffer, pH 7.3, to bring the total phosphate concentration to 0.009 M, 0.2 ml. of a mixture of 0.024 M MgCl₂ and 0.004 M MnCl₂, substrate neutralized with KOH at the indicated concentration, and solution KN to bring the final volume to 2.4 ml. The pH was 7.2 to 7.3. The center well contained 0.1 ml. of 10 per cent KOH. The rates given are those observed for the first 2 hours.

For ammonia determination the reaction was stopped by addition of 0.2 ml. of 3 N H₂SO₄ from the side arm. The determination was carried out on a 1 ml. aliquot by the method of Conway and Byrne (4).

For aspartic and glutamic acid determinations the reaction was also stopped by addition of 0.2 ml. of 3 N H₂SO₄. A 2 ml. aliquot of the mixture was neutralized to phenol red, used as internal indicator, heated for 10 minutes in a boiling water bath to deproteinize the suspensions, diluted

to 10 ml., and filtered. Suitable aliquots of the filtrate were used for microbiological assays. Glutamic acid was determined with *Lactobacillus arabinosus* 17-5, American Type Culture Collection No. 8014, and Medium D of Dunn *et al.* (5). For aspartic acid, *Leuconostoc mesenteroides* P-60, American Type Culture Collection No. 8042, and the medium described by Steele *et al.* (6) were used. In both cases the final volume was 5 ml. and included 2.5 ml. of double strength basal medium; the cultures were incubated in tubes at 32° for 72 hours and the extent of growth estimated by titration to brom thymol blue with 0.1 N NaOH. The salt concentration was made the same in all standard and unknown tubes by addition of the necessary volume of a solution of the same salt and indicator concentration as in the diluted reaction mixtures being analyzed. In order to insure that the samples to be analyzed contained no substances affecting the growth response of the organism, two different amounts of unknown and a mixture of unknown plus standard, all in duplicate and all in growth-limiting amounts, were included in each assay. In spite of these precautions, the accuracy of the analyses is probably no greater than ± 5 per cent.

Keto acid determinations were done on 10 per cent trichloroacetic acid filtrates of the vessel contents by the method of Lardy (7), except that spectrophotometric readings were taken at both 520 and 420 m μ . Both α -ketoglutaric acid and pyruvic acid standards were used and the concentration of each in the unknown sample was estimated from the absolute and relative values of the absorption at the two wave-lengths. The relative values differ markedly for the two acids (8). Oxalacetic acid would be included as pyruvic acid by this method, as reported by Friedemann and Haugen (8) and confirmed by us in control experiments.

In all cases measurements of initial as well as of final concentrations of reactants or products were made and the figures in Tables I, III, IV, and V represent the difference between these two values.

Results

Substrates—A variety of substances other than glutamate, pyruvate, and succinate, which were earlier shown to be oxidized by rickettsiae (1), have been found to produce no significant O₂ uptake by these organisms. These include fumarate, malate, citrate, butyrate, sucrose, glucose, and all of the naturally occurring amino acids except glutamate. With oxalacetate there was a low oxygen uptake, equal to that with pyruvate and probably due to spontaneous formation of pyruvate from oxalacetate. With α -ketoglutarate there was a low variable O₂ uptake, always greater than the uptake with no substrate. The rate was usually between 8 and 15 per cent of that with glutamate, but occasional preparations had rates well outside of this range. Examples that include the extremes of varia-

tion encountered are given in Column 4 of Table II. Attempts were made to confirm this apparent slow utilization of α -ketoglutaric acid by measurement of substrate disappearance. However, after 4 to 6 hours incubation

TABLE I

Utilization of Pyruvate and α -Ketoglutarate by Murine Typhus Rickettsiae

Substrate	O ₂ uptake	Change in	
		Pyruvate	α -Ketoglutarate
	μM	μM	μM
Pyruvate	1.78	-1.39	+0.01
α -Ketoglutarate	1.54	+0.09	-0.27

The initial amounts of pyruvate and α -ketoglutarate present were 2.10 and 2.31 μM respectively.

TABLE II

*Oxidation of Mixtures of α -Ketoglutarate and Aspartate or Alanine by Epidemic and Murine Typhus Rickettsiae**

Strain*	Preparation No.	Concentration of α -ketoglutarate	Rate of O ₂ uptake						
			No substrate	α -Ketoglutarate	Aspartate	α -Ketoglutarate + aspartate	Alanine	Alanine + α -Ketoglutarate	Glutamate
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
		M	$\mu l.$ per hr.	$\mu l.$ per hr.	$\mu l.$ per hr.	$\mu l.$ per hr.	$\mu l.$ per hr.	$\mu l.$ per hr.	$\mu l.$ per hr.
Murine	621	0.008	0.9	8.2		45			49
"	119	0.004	0.8	6.2	2.8	27			44
"	53	0.001	0.6	4.5	2.7	29	5.4	12	128
"	48	0.001	2.0	6.5		25			75
"	615†	0.008				36			101
Epidemic	511	0.007	2.1	4.6	1.8	41			65
"	41	0.008	7.5‡	13	7.8	51	9.8	37	57

The concentration of aspartate and alanine in the mixtures was 0.004 M. The concentration of glutamate was 0.014 M.

* The Breinl strain of epidemic and the Wilmington strain of murine typhus.

† This preparation of rickettsiae had been purified with albumin. The others had been subjected to one purification cycle only.

‡ The relatively high rate of O₂ uptake in the absence of substrate observed with this pool was rarely found with other preparations.

of rickettsiae with 0.001 M α -ketoglutarate, the change in α -ketoglutarate was so small as to be barely significant. Under similar conditions pyruvate disappearance was readily measurable, although the rate of O₂ uptake was of the same low order of magnitude. An example of the results of one

experiment in which pyruvate and α -ketoglutarate utilization were both determined after 5 hours incubation with a preparation of murine rickettsiae is given in Table I. From these results it is only possible to conclude that α -ketoglutarate is utilized slightly if at all. The O_2 uptake, although apparently significant, is consequently difficult to explain.

There was found one system other than glutamate which led to a fairly rapid O_2 uptake by the rickettsiae. This was a mixture of α -ketoglutarate and aspartate. Formation of glutamate by transamination and its subsequent oxidation seemed the most likely explanation for this observation. As can be seen from the data given in Table II, the rate with α -ketoglutarate and aspartate together was always greater than the sum of the rates with each alone, and varied between 30 and 90 per cent of the rate with glutamate. This variation in the ratio of the rate of O_2 uptake with glutamate to that with α -ketoglutarate and aspartate could be accounted for by the presence in variable amounts of a transaminase outside the rickettsial cell, as discussed later.

The other amino acids, with the exception of histidine and alanine, led to no increased O_2 uptake when added to α -ketoglutarate. The increase brought about by histidine proved to be a non-enzymatic reaction that took place as readily in the absence of rickettsiae as in their presence. The results with alanine and α -ketoglutarate were variable. The rate was in no instance as high as the rate with aspartate and α -ketoglutarate and frequently it was hardly significantly higher than the rate with α -ketoglutarate alone. Confirmatory evidence for the presence of an alanine transaminase, such as was later obtained for the aspartate-glutamate transaminase, could not be obtained.

Products of Oxidation of Glutamate—As a first step in determining the ultimate products of glutamate oxidation, simultaneous measurement was made of glutamate disappearance, oxygen consumption, and CO_2 and NH_3 formation (Table III). From the low value of the O_2 - Δ -glutamate ratio it was apparent that the oxidation was incomplete, although, since CO_2 was produced, there must have been some breakdown of the carbon chain. The great disparity between ammonia production and glutamate utilization suggested the accumulation of some nitrogen compound. In view of this and of the above indication of the presence of a transaminase, the reaction mixtures were subsequently analyzed also for aspartic acid. A considerable amount of this amino acid was found and the sum of the ammonia and aspartate formed was equal to the glutamate that disappeared, within the limits of error of the microbiological methods. One explanation for the formation of aspartate would be the oxidation of glutamate through α -ketoglutarate to oxalacetate, by the well known series of reactions that take place in mammalian tissue homogenates (9), followed by

reaction between a 2nd molecule of glutamate and the oxalacetate to give α -ketoglutarate and aspartate.

If the α -ketoglutarate were then oxidized only as far as oxalacetate, with subsequent transamination of the oxalacetate, it should be possible to convert glutamate to aspartate with formation of only a trace of ammonia. However, ammonia is formed to the extent of 30 to 45 per cent of the glutamate N. Therefore it must be assumed either that the rate of oxidation of α -ketoglutarate to oxalacetate is slower than the formation of aspartate from oxalacetate or that some of the oxalacetate undergoes

TABLE III
Products of Oxidation of Glutamate by Typhus Rickettsiae

	Epidemic (<i>R. prowaseki</i>)		Murine (<i>R. mooseri</i>)	
	Experiment I	Experiment II	Experiment III	Experiment IV
	μM	μM	μM	μM
Glutamate, initial	7.01	13.6	14.4	33.6
Δ -Glutamate	4.52	4.99	6.76	
O ₂	7.35	8.88	12.2	28.5
CO *	6.13		10.9	
NH ₃	1.74	2.28	2.14	4.19
Aspartate		2.98	4.74	12.6
NH ₃ + aspartate		5.26	6.98	
Keto acid		0.08	0.04	
O ₂ - Δ -glutamate ratio	1.6	1.8	1.8	
O ₂ -NH ₃ ratio	4.2	3.9	5.7	6.8
O ₂ -aspartate ratio		3.0	2.6	2.2

The reaction time was 3 hours in Experiments I and III, and 5 hours in Experiments II and IV.

* The value for CO₂ was calculated, as described in the text, from the observed manometric readings and the ratio between NH₃ formed in the flask containing KOH, given in the table, to that in the alkali-free flask. In Experiment I, the NH₃ formed in the alkali-free flask was 1.54 μM and Δ -glutamate was 4.07 μM . In Experiment III, the NH₃ formed in the alkali-free flask was 1.95 μM .

further oxidation. Actually the observed ratios of O₂ consumption to aspartate and NH₃ formation and to glutamate utilization can best be explained by a combination of these circumstances; that is, some complete oxidation and the accumulation of one or more intermediates prior to oxalacetate. This interpretation is in agreement with the finding in Experiment III (Table III) that CO₂ production exceeds the sum of aspartate and NH₃ formation, but is less than the amount required for complete oxidation of the glutamate not accounted for as aspartate. There is no evidence as to the nature of the intermediate, except that no appreciable

amount of keto acid accumulates under these conditions. There is an indication of a slight quantitative difference in end-products with the epidemic and murine strains.

It might be mentioned that, in spite of the slow rate of O_2 uptake with pyruvate, which is about 10 per cent (8 to 12 per cent) of that with glutamate, this substance cannot be excluded as an intermediate in the oxidation of glutamate. In view of the accumulation of aspartate and possibly of smaller amounts of some other intermediate, it is not necessary to assume that more than 1 out of every 6 to 10 molecules of glutamate metabolized is oxidized via pyruvate to account for the balances observed in Experiments II and III (Table III).

The products of the oxidation of pyruvate have not yet been determined, owing in part to its slow rate of utilization. Prolongation of the reaction time beyond 5 hours is not possible because of loss of activity (and viability) of the rickettsiae, and the supply of rickettsiae has not been sufficient to allow the use of higher concentrations. A few experiments similar to the one in Table I have shown that between 1 and 2 moles of O_2 are used per mole of pyruvate. The R. Q. has been found to be between 1.3 and 1.4. Thus this substrate also is incompletely oxidized. However, it is unlikely that its oxidation ceases with acetate formation, as is the case with certain bacteria (10, 11), since with the latter reaction the O_2 consumption would be only 0.5 mole per mole of pyruvate, and the R. Q. would be 2.0.

Transaminase—The presence of a transaminase was suggested by the oxygen uptake observed with a mixture of α -ketoglutarate and aspartate and by the formation of aspartate during the oxidation of glutamate. If, as postulated later, the rickettsiae are impermeable to various non-nitrogenous dicarboxylic acids, it would seem that aspartate formation from glutamate could best be explained by an intracellular transaminase and the oxidation of α -ketoglutarate plus aspartate by the presence of an extracellular transaminase. Both reactions could readily be explained by assuming that transaminase is normally present in rickettsiae, that it remains active in the non-viable organisms that are presumably present to a greater or less extent in all preparations of rickettsiae, and that these non-viable organisms are permeable to dicarboxylic acids. However, the further possibility exists that at least the "extracellular" transaminase activity was due to the contaminating fraction of yolk sac.

To test these possibilities, three reaction mixtures, containing aspartate and α -ketoglutarate with Mg, Mn, and phosphate as usual, were set up. To one were added 1.2 ml. of a suspension of viable rickettsiae that had been frozen for several days in sucrose-P, to a second 1.2 ml. of rickettsiae from the same pool that had been killed by freezing for several days in

salt K-7.5 (2), and to a third 1.2 ml. of the fraction obtained from normal yolk sac fractionated in the same way as the infected yolk sac pool. All were incubated for 3 hours at 34.3° in Warburg flasks, then analyzed for "pyruvate" and α -ketoglutarate. As mentioned above, oxalacetate is included as pyruvate in the method of analysis used; thus its spontaneous partial conversion to pyruvate would not confuse the results and the "pyruvate" found should be equivalent to the oxalacetate formed in the reaction.

The results shown in Table IV indicate that transamination did take place in all three mixtures. With viable rickettsiae, α -ketoglutarate disappearance exceeded the increase in "pyruvate," since the pyruvate,

TABLE IV

Changes in Keto Acid Concentration in Mixtures of α -Ketoglutarate and Aspartate

The mixtures were incubated for 3 hours with preparations from normal yolk sac and from yolk sac infected with typhus rickettsiae.

	Epidemic (<i>R. prowazeki</i>)		Fraction from normal yolk sac
	Sucrose-P*	Salt K-7.5*	Sucrose-P*
	μM	μM	μM
Δ , α -Ketoglutarate	-1.25	-1.27	-0.57
Δ - "Pyruvate"†	+0.96	+1.18	+0.52
O ₂	-1.63	-0.30	-0.2

The initial reaction mixtures contained 3.0 μM of α -ketoglutarate and 10 μM of aspartate.

* Medium in which rickettsiae or normal yolk sac fraction was suspended during freezing. Rickettsiae frozen in sucrose are largely viable; those frozen in salt K-7.5 largely dead.

† The figures for pyruvate include the oxalacetate formed in the transamination and any pyruvate formed by decarboxylation of the oxalacetate.

formed spontaneously from the oxalacetate produced, can be utilized by rickettsiae. With the fraction from normal yolk sac and with rickettsiae frozen in salt solution, neither of which can oxidize pyruvate (1), the changes in the two keto acids were equal within the limits of accuracy of the method, which is about $\pm 0.1 \mu M$ when dilution factors are taken into consideration. The extent of the reaction appeared to be greater in the infected than in the normal yolk sac preparations. But, although these findings were confirmed in a few other experiments, the differences between normal and infected yolk sac were not sufficiently large to prove with certainty whether or not the rickettsiae themselves have transaminase activity, in addition to that obviously present in the fraction from normal yolk sac. Further experiments were then undertaken with rickettsiae

from which most of the normal yolk sac constituents had been removed by treatment with albumin, as described above. Also the analytical procedure was changed. The rickettsiae were incubated with 0.014 M glutamate and 0.004 M oxalacetate, as the reaction is more rapid and more nearly complete in this direction (12). The mixture was incubated for 2 hours at 34.3° and then analyzed for aspartate, a more specific analysis than the keto acid analysis used in the first experiments. The results are shown in Table V. The latter also includes an experiment with one infected and one normal pool, prepared in the usual way, which does not completely remove yolk sac material. This confirms the results reported in Table IV.

With the albumin-treated preparations from normal yolk sac, aspartate formation from glutamate and oxalacetate was reduced to small, often negligible amounts. The albumin-treated rickettsiae, on the other hand, still formed aspartate, the richer pools, Preparations 625, 627, and 630, forming considerably more than Preparation 615, which was relatively poor in rickettsiae. With Preparations 627 and 630 the over-all amount of aspartate formed appeared to be independent of the viability of the rickettsiae, since it was not markedly changed by storage at -20° or by freezing in the absence of sucrose, although these procedures are known to reduce the viability of rickettsiae (2) and had greatly lowered their O₂ uptake. But if, as assumed, killing the rickettsiae results in increased permeability to oxalacetate, one might expect that the rate of aspartate formation would increase in proportion to the number of dead organisms, if the procedures used in killing the rickettsiae had been sufficiently mild. This, however, does not occur, because the viable rickettsiae can form aspartate directly from glutamate alone (presumably via oxalacetate formed by oxidation of glutamate) and this ability is lost when the rickettsiae are killed. This loss balances out any increased aspartate formation by the dead organisms due to increased permeability to oxalacetate.

With the two preparations that most rapidly oxidized glutamate, Preparations 625 and 630, aspartate formation actually decreased after the rickettsiae were killed. This was at least in part due to the limited quantity of oxalacetate added which was 10 μ M. The viable rickettsiae from these two pools produced 10 and 13 μ M of aspartate respectively, quantities that could not be formed by the corresponding non-viable preparations that were entirely dependent upon the limited amount of added oxalacetate. Actually, since the transamination reaction is reversible, it is possible that its rate was slowed down to some extent by approach to equilibrium with all of the non-viable preparations. With the quantities of reactants used in these experiments, assuming an equilibrium constant of 3.5 (12), equilibrium would be reached with the formation of 9 μ M of

aspartate, if the oxalacetate were stable. Since control experiments in the absence of rickettsiae indicated that about 50 per cent of the oxalacetate was decarboxylated in 2 hours, the equilibrium quantity of aspar-

TABLE V
Formation of Aspartate from Glutamate and Oxalacetate by Viable and Non-Viable Murine Typhus Rickettsiae

Strain	Preparation No.	Treatment	O ₂ uptake,* glutamate + oxalacetate	Aspartate formed		Aspartate formation due to added oxalacetate
				Gluta- mate	Gluta- mate + oxalace- tate	
(1)	(2)	(3)	(4)	(5)	(6)	(6)-(5)
Pools subjected to 1 purification cycle only						
Murine	67		μM 25.8	μM 9.76	μM 13.7	μM 3.94
"	67	Frozen in salt K-7.5	2.2	0.20	6.48	6.28
Normal	524		0		3.35	
"	524	Frozen in salt K-7.5	0		3.64	
Pools subjected to 1 purification cycle, then treated with albumin						
Murine	627	Frozen in sucrose	8.86	4.38	5.49	1.11
"	627	" " salt K-7.5	1.04		6.40	6.40†
"	625		14.8	7.96	10.1	2.14
"	625	Frozen in salt K-7.5	2.07		6.95	6.95†
"	630	Frozen in sucrose, stored -70°	7.74	3.42	5.90	2.48
"	630	Frozen in sucrose, stored -20°	0.19	0	5.72	5.72
"	615		2.40	1.25	1.86	
Normal	63	Frozen in sucrose	0.06		1.10	
"	625		0		0.20	
"	651		0.1		0.12	

The reaction mixtures contained initially 33.6 μM of glutamate and 10 μM of oxalacetate. The incubation period was 2 hours.

* The O₂ uptake was the same with or without oxalacetate.

† It was assumed that with these two killed preparations, as with Preparations 67 and 630, no aspartate was formed from glutamate alone.

tate would be less than 9 μM at this time. No such limitation would apply to the viable rickettsiae, which produce aspartate independently of added oxalacetate.

Thus several factors favor the production of relatively large over-all quantities of aspartate by the viable rickettsiae. It is, however, clear that preparations consisting largely of viable rickettsiae form considerably less aspartate from added oxalacetate than do those in which most of the rickettsiae have been killed (see the last Column, Table V). It might be suggested that the apparent transaminase of the rickettsiae, even after albumin treatment, is due to adsorption of the yolk sac enzyme. Such a possibility, however, seems unlikely in view of this greater effect of added oxalacetate after the rickettsiae have been killed, together with the fact that the yolk sac transaminase is unaffected by the procedures used in killing the rickettsiae.

From these results it must therefore be concluded that the rickettsiae themselves have a transaminase and that this enzyme can survive certain procedures that greatly reduce both the number of viable rickettsiae and their ability to oxidize glutamate. This could only be demonstrated after removal of the yolk sac transaminase from the usual preparations by the albumin treatment.

A few unsuccessful attempts have been made to detect in rickettsiae the presence of a phosphorylating mechanism similar to that associated in mammalian tissues with the oxidation of many substrates (12-18), including those known to be oxidized by rickettsiae. If rickettsiae are prepared in the absence of phosphate and used in the usual concentration, the total acid-soluble phosphate is barely detectable by the procedures used (19); thus it was impracticable to follow changes in endogenous phosphate fractions. In view of the apparent impermeability of rickettsiae to dicarboxylic acids, it also seemed improbable that addition of adenylic acid, hexokinase, glucose, phosphate, and fluoride, which make possible detection of phosphorylation by washed tissue particles (17, 18), would do so with this system. These additions did indeed prove ineffective.

There is, however, some evidence that phosphate may be required for glutamate oxidation by rickettsiae. If rickettsiae are subjected to a second cycle of purification in the absence of phosphate, with either solution KN-G or 6 per cent albumin, the rate of oxygen uptake without added phosphate is generally appreciably lower than the rate in its presence. A concentration of 0.01 M phosphate or higher seems to be required to achieve the maximum rate with such preparations (Table VI). On the other hand, with rickettsiae subjected to only one cycle of purification, in the absence of phosphate, the rate is quite independent of added phosphate, although the inorganic phosphate remaining in such preparations is only 10^{-4} M or lower. It is possible that rickettsiae are only slightly and slowly permeable to inorganic phosphate, as to other dibasic acids, and

that it is therefore difficult both to wash out their own internal phosphate and to replace this once it has been depleted.

Rickettsiae that are activated by phosphate show a decreased rate if Mg or Mn is omitted from the system, indicating that these ions also may be required.

When the rate of O₂ uptake is limited by lack of phosphate, some keto acids accumulate in the reaction mixture. This does not occur in the

TABLE VI

Effect of Phosphate, Mg, and Mn on Rate of O₂ Uptake of Murine Typhus Rickettsiae with Glutamate As Substrate

Experiment No.	Mg, 2×10^{-3} M	Mn, 3.3×10^{-4} M	Phosphate concentration $M \times 10^3$	O ₂ uptake $\mu\text{l. per hr.}$	Keto acid formed*	
					α -Ketoglu- tarate μM	"Pyru- vate" μM
46	+	+	0	83	0.32	1.20
	+	+	0.85	97	0.23	1.00
	+	+	2.8	108		
	+	+	8.5	118	0.16	0.07
228	+	+	0	47		
	+	+	10	97		
	+	—	10	66		
	—	+	10	71		
211	—	—	10	53		
	+	+	0	20		
	+	+	10	43		
	—	—	10	17		
629	+	+	0	8.3	0.36	0.13
	+	+	8.5	60	0.13	0.02
315	+	+	0	4.1		
	+	+	4	35		
	+	+	10	39		

The concentration of glutamate in all the experiments was 0.014 M. As in Table IV, the figures for "pyruvate" include both pyruvate and oxalacetate.

* After 4 hours incubation at 34.3°.

presence of sufficient phosphate, and may indicate that these acids are normally intermediates in the oxidation of glutamate and that their oxidation is particularly dependent upon an adequate concentration of phosphate.

DISCUSSION

It seems probable on the basis of the results reported here that rickettsiae oxidize glutamate by the same series of reactions that account for

its oxidation by mammalian tissue particles; that is, first to α -ketoglutarate and ultimately to CO_2 and water through the citric acid cycle. This is strongly suggested by the formation of aspartate during the oxidation of glutamate, which, together with the evidence for the presence of a glutamate-aspartate transaminase, implicates oxalacetate as an intermediate. Also, since there is no accumulation of the α -ketoglutarate formed in the transamination reaction, this substance is presumably oxidized by the rickettsiae. Pyruvate, another metabolite oxidized by this cycle in tissues and a possible intermediate in glutamate oxidation, is oxidized by rickettsiae. Finally succinate, a fourth intermediate in the cycle, is oxidized, though only slowly in preparations in which the majority of the rickettsiae are viable (1). There is still no evidence, however, for the occurrence of one portion of the citric acid cycle in rickettsiae, the formation and utilization of citric acid itself.

Also noteworthy is the lack or very slow rate of oxygen uptake by rickettsiae in the presence of many of the intermediates of the cycle such as fumarate, malate, and oxalacetate, α -ketoglutarate and citrate, which would be formed in the oxidation of glutamate if it proceeds by the suggested route. This can only be explained by the assumption mentioned earlier that they are impermeable to dicarboxylic acids. It is possible that they are slowly permeable to succinate, or that the oxidation of this acid is brought about only by the non-viable rickettsiae present, since the rate is greater after the rickettsiae have been killed by freezing in isotonic salt solution (1). Neither glutamate nor any of the other postulated intermediates are oxidized by rickettsiae that have been killed in this way, with or without addition of adenylic acid. This parallels the situation in tissue homogenates, in which succinoxidase appears to be more stable than the other oxidative enzymes (20). Most of the oxidative enzymes of the tissues are destroyed by freezing under the conditions that lead to loss in ability of rickettsiae to oxidize glutamate, and their destruction can also be prevented by the same precautions of rapid freezing in the presence of sucrose and storage at -70° that protect the rickettsiae (21).

With washed tissue particles, the functioning of the citric acid cycle requires the presence of Mg, phosphate, and adenylic acid, as well as a suitable substrate (15, 18). Under certain conditions it can be shown that the oxygen uptake of rickettsiae also is increased by phosphate and Mg, but it has never been possible to demonstrate any effect with adenylic acid. However, if they are impermeable to dicarboxylic acids, they probably are also impermeable to adenylic acid. The quantity of rickettsiae available has not yet been sufficient to warrant an attempt to detect the presence of this or of any other coenzyme.

One of the most notable characteristics of the oxidations of the citric

acid cycle in tissues is the linked formation of high energy phosphate bonds (13-18), which may be the means by which these oxidative reactions furnish energy to the cell (22). It would naturally be of especial interest to know whether phosphorylation also accompanies the oxidations that take place in these obligate intracellular organisms. The fact that under certain conditions glutamate oxidation by these organisms, as by washed tissue particles, is dependent upon the presence of inorganic phosphate may be an indication, though certainly not conclusive evidence, of a phosphorylation-linked oxidation. Real evidence for or against this, as well as for the ability of rickettsiae to oxidize many of the intermediates of the citric acid cycle, must await the separation of the oxidative enzymes from viable rickettsiae and hence from the complicating permeability factors that are encountered so often in dealing with living cells.

SUMMARY

1. Typhus rickettsiae show no significant O_2 uptake with fumarate, malate, oxalacetate, citrate, butyrate, or with any amino acid other than glutamate. There is a low oxygen uptake with α -ketoglutarate, but utilization of this compound could not be confirmed by measurement of change in substrate concentration. There is, however, a rapid oxygen uptake with a mixture of α -ketoglutarate and aspartate.

2. During the oxidation of glutamate, aspartate and ammonia are formed. These two compounds account for all of the nitrogen of the glutamate that is utilized.

3. Transaminase is present in the yolk sac fraction of the usual rickettsial preparations. This fraction can be removed by agglutination in 6 per cent serum albumin. With rickettsiae that have been purified in this way and killed by freezing, it is possible to show the existence of a rickettsial transaminase that remains active after loss of viability and of oxidative enzymes.

4. The observations presented suggest that oxidation of glutamate by rickettsiae proceeds through the citric acid cycle, with formation of aspartate as a by-product due to the presence of transaminase, and that these organisms are impermeable to the dicarboxylic acid intermediates of the cycle.

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THE METABOLISM OF 16-KETOESTRONE AND 16-KETO- α -ESTRADIOL IN MAN*

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We have reported (1) the isolation and identification of estriol as one of the estrogenic urinary excretion products from the injection of a massive dose (600 mg.) of 16-ketoestrone in a man, thus strengthening the hypothesis (2) that 16-ketoestrone is an intermediate in the *in vivo* conversion of estrone to estriol. According to this hypothesis 16-ketoestrone is first converted into 16-keto- α -estradiol by the addition of 1 molecule of hydrogen and subsequently reduced to estriol by the addition of another molecule of hydrogen. The isolation of additional estrogenic urinary excretion products was not attempted at this time. It is the purpose of the present communication to describe a series of studies on the metabolism of 16-ketoestrone and 16-keto- α -estradiol in men, which indicate not only the resulting urinary excretion of estriol from each of these compounds but also the urinary excretion of another product which gave a typical Kober color test (3) for estrogens only when zinc dust had been added during hydrochloric acid hydrolysis of the urinary estrogens. This product appeared preponderantly in the estradiol fraction obtained by our liquid chromatogram technique (4) for the estrogens. Similar observations have been reported (5) on human male urine to which 16-ketoestrone was added immediately preceding zinc-hydrochloric acid hydrolysis. The studies with exogenous estrogens (16-ketoestrone and 16-keto- α -estradiol) to be described therefore provide a working model in support of the hypothesis of Smith and Smith (6) that more highly oxidized estrogens than those presently known (estrone, α -estradiol, estriol), which may be converted to more active forms during acid hydrolysis by the addition of zinc dust are excreted in the urine.

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EXPERIMENTAL

Men without any evidence of organic disease were injected intramuscularly with the steroids (16-ketoestrone and 16-keto- α -estradiol) dissolved in propylene glycol (20 mg. per cc.). The complete 48 hour postinjection urine specimen was collected with toluene as a preservative. Within 2 days after completion of the collection period, aliquot parts of each specimen were processed, chromatographed, and titrated by previously described methods (4). Color correction equations similar to the one described by one of us (7) for the Kober reagent were utilized for the color tests made on urinary residues in these studies. The Kober (3), Zimmermann (8), and

TABLE I

Photometric Density ($-\log T$) of Color Products Formed by 16-Ketoestrone and 16-Keto- α -estradiol

	No. of determinations	Reagent	Average photometric density*		Photometric density at 520 m μ relative to estrone, 1.0
			L_{430}	L_{490}	
50 γ 16-ketoestrone	2	Kober	0.025	0.026	0.06
	2	Zimmermann	0.011	0.012	0.04
	2	Bachman	0.085†	0.110	0.25‡
50 " 16-keto- α -estradiol	6	Kober	0.509	0.112	1.95§
	2	Zimmermann	0.027	0.021	0.09
	2	Bachman	0.059†	0.156	0.18

* Evelyn photoelectric colorimeter.

† 540 m μ .

‡ Relative to estriol, 1.0.

§ Relative to α -estradiol, 1.0.

Bachman (9) color tests and the Evelyn photoelectric colorimeter were employed as previously described (4).

RESULTS AND DISCUSSION

Colorimetric Data on Pure 16-Ketoestrone and 16-Keto- α -estradiol—The data summarized in Table I show that 16-ketoestrone and 16-keto- α -estradiol do not react typically with either the Zimmermann or the Bachman reagent. With the latter reagent a faint fluorescent yellowish pink color is produced by both of these compounds. This accounts for the photometric density reading at 540 m μ which is 25 per cent of that produced by estriol in the case of 16-ketoestrone and 18 per cent in the case of 16-keto- α -estradiol. With the Kober reagent, 16-ketoestrone was essentially non-chromogenic, whereas 16-keto- α -estradiol developed an initial yellowish orange color which became typically pink on dilution with

an equal volume of water and reheating. These are in agreement with Marlow's published observation (10). The spectral ratio, 4.5 (L_{520}/L_{420}), for the final Kober color product was somewhat lower than has been reported (7) for estrone, 10, α -estradiol, 7.5, and estriol, 10.2, whereas the photometric density per microgram at 520 $m\mu$, 0.0102, was slightly higher than has been reported (7) for these estrogens. Ultraviolet absorption curves (230 to 300 $m\mu$) of 16-ketoestrone and 16-keto- α -estradiol in 95 per cent ethyl alcohol (Beckman DU quartz spectrophotometer) were found to be typical of published curves for estrone, α -estradiol, and estriol (11).

Colorimetric Urinary Estrogen Excretion Data Following Injection of 16-Ketoestrone in Men—Data on the estrogens found in the 48 hour post-injection urine of men treated with single intramuscular injections of 60 mg. of 16-ketoestrone are summarized in Table II. Simple hydrochloric acid hydrolysis of Aliquot 1 of Experiment A, boiled 20 minutes, developed a typical Kober color only in the 30 per cent methanol-benzene (estriol) filtrate fraction of our liquid chromatogram. Aliquot 2 which was boiled for a longer period (3 hours) in order to test the completeness of hydrolysis yielded essentially similar results. Furthermore, it was observed that the initial yellow Kober color of the estriol fraction did not develop instantly in the boiling water bath, as is characteristic of estrone, α -estradiol, and 16-keto- α -estradiol, but sluggishly, as is characteristic of estriol, and that the relatively high spectral ratio of the final product ($L_{520}/L_{420} = 5.4$ to 8.9) was more typical of urinary estriol residues than of estradiol residues (4).

The estrogen titers derived from Aliquot 3, which was boiled for 3 hours after the addition of 15 volumes per cent of concentrated hydrochloric acid and 4 gm. per cent of zinc dust, are especially noteworthy. In addition to estriol in the 30 per cent methanol-benzene chromatographic fraction, a residue was obtained in the 5 per cent methanol-benzene fraction which produced the typical Kober color. An aliquot of this residue was essentially negative by the Zimmermann test; thus estrone could not account for the estrogen titer. If the Kober color product produced by the 5 per cent methanol-benzene fraction is calculated as estradiol, it constitutes a 14-fold increase in estrogen titer over that obtained in the same fraction by simple hydrochloric acid hydrolysis. This material develops the initial yellow color instantly on heating in contrast to estriol which, as has been mentioned, develops the initial yellow color sluggishly. Furthermore, the spectral ratio, 2.5, of the final pink color is more typical of 16-keto- α -estradiol, 4.5, than of estriol, 10. We have shown that 16-ketoestrone, which is non-chromogenic with the Kober reagent, added to male urine just before zinc-hydrochloric acid hydrolysis yielded products

in our 5 per cent and 30 per cent methanol-benzene chromatographic fractions (approximately 2:1) which gave the characteristic Kober color. These results therefore would seem to suggest that 16-ketoestrone is in

TABLE II

Colorimetric Urinary Estrogen Excretion Data on Men after Injection of 16-Ketoestrone (60 Mg.) (48 Hour Postinjection Specimen)

Experiment	Aliquot ($\frac{1}{2}$ total) No.		Color test	Estrogen titer of chromatographic fractions, γ per aliquot		
				2 per cent methanol- benzene, estrone	5 per cent methanol- benzene, estradiol	30 per cent methanol- benzene, estriol
A	1	Boiled 20 min. with 10 vol. % concen- trated HCl	Kober	12 0.78*	43 0.98*	1090 5.3*
	2	Boiled 3 hrs. with 10 vol. % concen- trated HCl	"	4 0.62*	4 0.61*	1154 6.5*
	3	Boiled 3 hrs. with 15 vol. % concen- trated HCl and 4 gm. % zinc	"	17 0.19*	619 2.6*	1530 8.9*
			Zimmermann	10	5	10
	1	Boiled 20 min. with 10 vol. % concen- trated HCl	Kober	14 0.75*	24 0.81*	1200 6.3*
			Bachman	0	0	1100
B	1	Reboiled 3 hrs. with 15 vol. % concen- trated HCl and 4 gm. % zinc	Kober	28 1.8*	380 2.1*	102 3.7*
	2	Boiled 3 hrs. with 10 vol. % concen- trated HCl	"	10 0.71*	20 0.82*	1136 6.8*
			Bachman	No test	No test	1050
	3	Boiled 3 hrs. with 15 vol. % concen- trated HCl and 4 gm. % zinc	Kober	101 2.1*	808 3.6*	1600 9.0*
			Zimmermann	Negative	No test	No test
			Bachman	No test	Negative	1400

* Spectral ratio L_{520}/L_{420} of Kober color product (Evelyn photoelectric colorimeter).

part excreted unchanged (except for conjugation) and is responsible by reduction to 16-ketoestradiol or estradiol for the greatly augmented Kober titers obtained in the 5 per cent methanol-benzene residue by zinc-hydrochloric acid hydrolysis.

In Experiment B, the Bachman reagent, which is essentially specific

for estriol, was utilized wherever possible in order to provide additional evidence for the identity of the estrogenic material appearing in our 5 per cent and 30 per cent methanol-benzene chromatographic filtrate fractions. With the residue in the 30 per cent methanol-benzene chromatographic fraction the Bachman reagent yielded the typical purple color and the titers derived therefrom were in close agreement with the Kober reagent product. This supports the concept that estriol accounts for all of the Kober color yielded by this fraction. On the other hand, the residue in the 5 per cent methanol-benzene chromatographic fraction of Aliquot 3 which gave a typical Kober color equivalent to 808 γ (of α -estradiol) developed only a faint fluorescent yellowish pink color with the Bachman reagent. The titer obtained from Aliquot 1 by zinc-hydrochloric acid hydrolysis subsequent to simple hydrolysis and extraction would seem to indicate that the metabolite in the urine (presumably 16-keto-estrone) responsible for the augmented estrogen titers following zinc-hydrochloric acid hydrolysis is excreted in conjugated form and is fairly resistant to simple hydrolysis with 10 volumes per cent of hydrochloric acid. We have found¹ that these hydrolytic conditions are adequate for the quantitative hydrolysis of sodium estrone sulfate added to pooled male urine. Therefore a type of conjugation more resistant to hydrolysis than the sulfate seems to be indicated.

We are unable to account for the small amount of estrogen (101 γ) in the 2 per cent methanol-benzene fraction of Aliquot 3 as indicated by the Kober reagent, since the residue did not give a characteristic Zimmermann color and therefore cannot be estrone. It perhaps is identical with the material appearing in much larger amounts in the 5 per cent methanol-benzene fraction.

Colorimetric Urinary Estrogen Excretion Data Following Injection of 16-Keto- α -estradiol in Men—Simple hydrochloric acid hydrolysis of an aliquot of the 48 hour urine specimen collected after injection of 10.0 mg. of 16-keto- α -estradiol in a man (Aliquot 1, Experiment A, Table III) yielded significant² amounts of estrogen in only the 30 per cent methanol-benzene (estriol) chromatographic fraction. This material in the 30 per cent methanol-benzene fraction gave a typical Bachman color and yielded titers which were in agreement with those derived by the Kober reagent. Estriol must therefore be one of the estrogenic urinary excretion products of 16-keto- α -estradiol in man. Since no significant amount of estrogen was found by the Kober reagent in the 5 per cent methanol-benzene

¹ Unpublished data.

² We have shown (7) that the use of a color correction equation and the Kober reagent involves an uncertainty factor of ± 7 γ of estrone, ± 10 γ of estradiol, and ± 4 γ of estriol for 24 hour titers.

TABLE III
*Colorimetric Urinary Estrogen Excretion Data after Single Injection
 of 16-Keto- α -Estradiol*

Experiment	Aliquot No.	Nature of hydrolysis	Estrogen titer, γ per aliquot of chromatographic fractions			
			2 per cent methanol-benzene, estrone	5 per cent methanol-benzene, estradiol	30 per cent methanol-benzene, estriol	
			Kober	Kober	Kober	Bachman
A. 10 mg. injection	1*	Boiled 20 min. with 10 vol. % concentrated HCl	9	0	185	130
	2*	Boiled 3 hrs. with 15 vol. % concentrated HCl and 4 gm. % zinc	12	108	411	330
B	1†	Boiled 20 min. with 10 vol. % concentrated HCl	2	3	79	96
	1†	Reboiled 3 hrs. with 15 vol. % concentrated HCl and 4 gm. % zinc	1 (3)†	30 (33)†	76 (155)†	80 (176)†
	2†	Boiled 1 hr. with 10 vol. % concentrated HCl	4	4	137	138
	2†	Reboiled 2 hrs. with 15 vol. % concentrated HCl and 4 gm. % zinc	2 (6)	20 (24)	30 (167)	No test
	3†	Boiled 20 min. with 15 vol. % concentrated HCl	4	4	134	No test
	3†	Reboiled 3 hrs. with 15 vol. % concentrated HCl and 4 gm. % zinc	0 (4)	16 (20)	37 (171)	" "
	4†	Boiled 3 hrs. with 15 vol. % concentrated HCl and 4 gm. % zinc	32	64	191	152

* One-half total.

† One-fourth total.

‡ The figures in parentheses represent the total of the two figures immediately above.

chromatographic fraction, it is unlikely that more than traces of 16-keto- α -estradiol were excreted unchanged. The significant yield of estrogen found in the 5 per cent methanol-benzene chromatographic fraction following zinc-hydrochloric acid hydrolysis of an aliquot of the urine (Aliquot 2, Experiment A, Table III) would seem to indicate that 16-ketoestrone also was an excretion product from the *in vivo* metabolism of 16-keto- α -estradiol and that its reduction has occurred during zinc-hydrochloric acid hydrolysis. In Experiment B (Table III) in which sequential simple and zinc-hydrochloric acid hydrolysis and extractions were performed, the presence of typical Kober products in the 5 per cent methanol-benzene (estradiol) chromatographic fraction following zinc-hydrochloric acid hydrolysis, even when simple hydrolysis and extraction had

TABLE IV

Recovery of Urinary Estrogens; Per Cent of 16-Ketoestrone and 16-Keto- α -estradiol Injected

Experiment		Hydrolysis	5 per cent methanol-benzene, estradiol	30 per cent methanol-benzene, estriol
A	60 mg. 16-ketoestrone	Simple		7.7
		Zinc-HCl	2.7	10.0
B	60 " "	Simple		5.5
		Zinc-HCl	3.8	7.6
C	10 " 16-keto- α -estradiol	Simple		3.7
		Zinc-HCl	2.2	8.2
D	10 " "	Simple		5.5
		Zinc-HCl	3.8	7.6

been performed, offered evidence of the presence of a reducible metabolite rather than of protection from atmospheric oxygen of a highly labile one.

On a percentage basis (Table IV) 16-ketoestrone and 16-keto- α -estradiol injected into man do not appear from these studies to yield significantly higher urinary estriol titers than have been reported for similar injections of estrone (12, 13); also a major fraction of the injected material remains to be accounted for. However, these considerations do not vitiate the observation that man does possess the capacity to convert 16-ketoestrone and 16-keto- α -estradiol to estriol and that these compounds may therefore be intermediates in the *in vivo* conversion of estrone to estriol.

The additional urinary metabolite of 16-ketoestrone and 16-keto- α -estradiol which becomes chromogenic with the Kober reagent only when zinc dust has been added during hydrochloric acid hydrolysis of the es-

trogen never exceeded 4 per cent (calculated as α -estradiol) of the injected compound or approximately one-half as much as was recovered as estriol. Its identity cannot be established by these studies. The conversion of 16-ketoestrone to 16-keto- α -estradiol by nascent hydrogen might satisfactorily account for the greatly augmented (14- to 60-fold) estrogen titers indicated by the Kober reagent in our estradiol fraction when zinc dust has been added during hydrolysis of the urinary estrogens. However, more rigorous fractionation studies³ in which 16-ketoestrone has been added to pooled male urine immediately preceding zinc-hydrochloric acid hydrolysis indicate that the material reactive with the Kober reagent (a) cannot be 16-ketoestradiol since it appears in the non-ketonic fraction on treatment with Girard's Reagent T, (b) is alcoholic since it appears in the alcoholic fraction upon treatment with phthalic anhydride, and (c) is not estriol since it does not react typically with the Bachman reagent. Heard and Saffran (14) have reported 17-desoxyestrone to be the major hydrolytic product following zinc-hydrochloric acid hydrolysis of sodium estrone sulfate. It may well be that 16-ketoestrone loses its oxygen function at C-16 while the C-17 carbonyl group undergoes reduction, so that estradiol is responsible for the Kober color in our 5 per cent methanol-benzene chromatographic fraction following zinc-hydrochloric acid hydrolysis. Such a mechanism would more satisfactorily account for the augmented bioassays in human urine obtained by Smith and Smith (6).

SUMMARY

Colorimetric urinary estrogen excretion data on men after injection of 16-ketoestrone and 16-keto- α -estradiol indicate that man is capable of converting these compounds to estriol but not to estrone. There is indirect evidence that 16-ketoestrone appears in the urine following injection with either of these compounds and that reduction of it by zinc-hydrochloric acid hydrolysis of its conjugate in urine accounts for the augmented estrogen titers obtained by this method of hydrolysis.

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DEUTERIUM STUDIES IN NORMAL MAN

I. THE RATE OF SYNTHESIS OF SERUM CHOLESTEROL

II. MEASUREMENT OF TOTAL BODY WATER AND WATER ABSORPTION*

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The rate of synthesis of serum cholesterol may be measured by determining the rate at which deuterium is incorporated into the serum cholesterol when an elevated deuterium concentration is maintained at a steady level in the body water. The rise in deuterium concentration in the serum cholesterol represents the appearance in the circulation of newly synthesized cholesterol molecules. The labeled cholesterol is formed from precursors which have derived their deuterium from the labeled body water (1).

Previous studies have shown that about half of the stably bound hydrogen atoms of cholesterol synthesized in the body are derived from body water. When non-isotopic cholesterol is present in the diet, it will dilute the deuteriocholesterol formed in the body and result in a lower maximum isotope concentration in the serum cholesterol. This maximum isotope concentration is ascertained experimentally by determining the maximum isotope concentration which is reached on prolonged maintenance of a steady deuterium concentration in the body water. The time required to reach 50 per cent of this maximum deuterium concentration is the half life time, $t_{\frac{1}{2}}$, of serum cholesterol, *i.e.* the time required for half the serum cholesterol molecules present in the circulation to be synthesized. In an individual in the steady state, the rate of cholesterol synthesis is equal to the rate of cholesterol degradation. Consequently, $t_{\frac{1}{2}}$ is also the time required for the disappearance of half the serum cholesterol molecules which have been formed at any one time.

This report is concerned with the study of the rate of synthesis (and degradation) of serum cholesterol in a normal man. Previous studies in mice have revealed a $t_{\frac{1}{2}}$ for total body cholesterol of 15 to 25 days (1). By maintaining a steady concentration of deuterium in the body water and by measuring the time required for the serum cholesterol to attain 50 per cent of the maximum concentration of deuterium, a value of about 8 days for

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the half life time of labeled serum cholesterol in the subject has been obtained (Fig. 1). The rate of appearance of labeled serum cholesterol depends on the rate of synthesis of cholesterol in the liver and other tissues and on the rate of transfer to the serum from these organs. Since the rate of transfer from the organs to the serum is most likely very rapid, the rate of appearance of labeled serum cholesterol should be a close reflection of the rate of synthesis of cholesterol in these organs.

A value of $t_{\frac{1}{2}}$ of 8 days represents a turnover time, $\bar{T}$, of about 12 days ($\bar{T} = t_{\frac{1}{2}}/\ln 2$) when, as in this case, the logarithm of the isotope concentration is a linear function of time. The turnover time is the mean survival

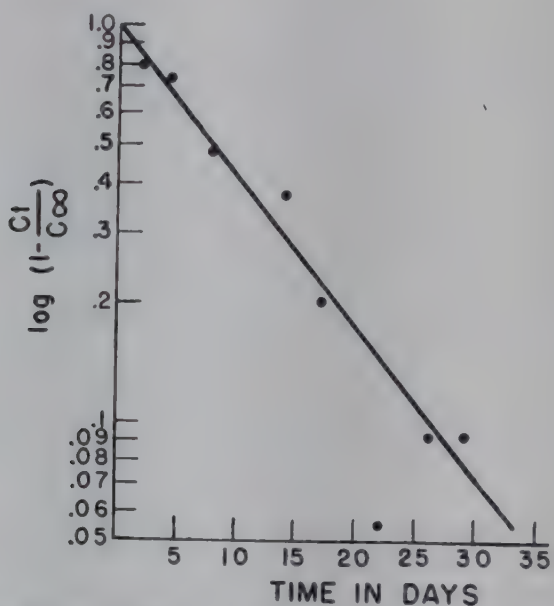


FIG. 1. Deuterium concentration in serum cholesterol digitonide plotted semi-logarithmically. C_t = deuterium concentration at any time t . C_{∞} = maximum deuterium concentration.

time for a serum cholesterol molecule. $\bar{T}$ is related to the rate at which serum cholesterol molecules are synthesized. This relationship is given by the equation $\bar{T} = M/m$, in which M is the total mass of circulating serum cholesterol and m is the mass of serum cholesterol synthesized per day. The plasma volume of this subject was 3750 ml., and the serum cholesterol level averaged 175 mg. per 100 ml. of serum. M accordingly was 6.56 gm. and m 546 mg. per day.

The administration of D_2O by mouth afforded an opportunity for observations on the absorption of water from the alimentary tract and on the estimation of total body water. The first dose of D_2O was given as 450 ml. of a 10 per cent D_2O solution. Blood samples were drawn from the antecubital veins during the following hour and deuterium analyses of the serum water were performed. The results are plotted in Fig. 2.

The rapid absorption of water from the alimentary tract is indicated by the appearance of D_2O in the venous blood shortly after ingestion. Extrapolation of the curve to the abscissa indicates that the first appearance of ingested water occurs about 7 minutes after ingestion. The rising curve is a reflection of continued absorption which appears to be completed within about 40 minutes. The data suggest that equilibration of serum water with the remaining body water keeps pace with absorption from the intestine; if there were a lag between absorption and equilibration, a decline in deuterium concentration following the completion of absorption on about

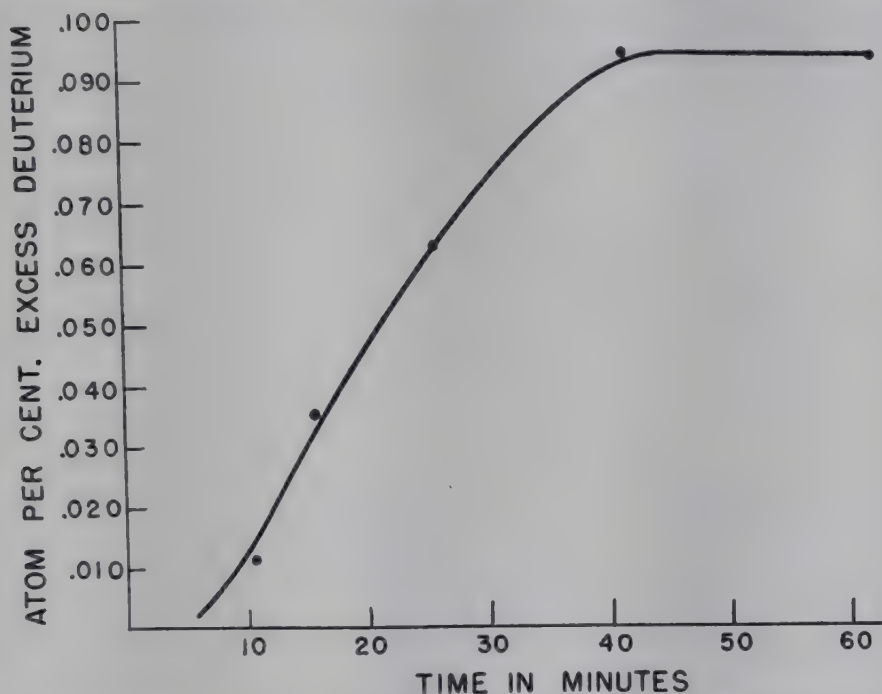


FIG. 2. Deuterium concentration in serum water following the ingestion of 450 ml. of a 10 per cent D_2O solution.

the 40th minute should have occurred. The absence of such a decline indicates very rapid equilibration following absorption of water.

The deuterium concentration in the serum water, once equilibration with the remaining body water has occurred, permits the calculation of total body water by the principle of isotope dilution. This principle has been used by Hevesy and Hofer (2) and by Moore (3) for measurements of total body water. The former administered D_2O by mouth and considered the isotope concentration in the urine after the 1st day to be identical with the body water concentration; calculations from their data yielded a value for total water volume of 43 ± 3 liters or 63 ± 4 per cent of the body weight. Moore injected D_2O intravenously and found that complete equilibration occurs within an hour; the deuterium concentration in the 1 hour plasma sample could be considered equivalent to the deuterium concentration in

total body water. This method yielded a value of 47.8 liters or 72 per cent of the body weight in a normal adult.

The deuterium concentration in the serum water of our subject at equilibrium was 0.094 atom per cent excess deuterium. 450 ml. of a solution of D_2O containing 10.0 atom per cent excess deuterium were given. By the principle of isotope dilution the product of the isotope concentration of a substance and the volume of the isotopic substance which is mixed with similar non-isotopic substance equals the product of the isotope concentration in the mixture and the volume of the mixture. In this case, $450 \text{ ml.} \times 10.0 \text{ atom per cent excess D} = \text{total body water} \times 0.094 \text{ atom per cent excess D}$. The total body water is 47.84 liters, or 71.8 per cent of the body weight (66.7 kilos). It is to be noted that in this experiment total ionizable

TABLE I

Deuterium Concentration (Atom Per Cent Excess D) in Body Water and in Serum Cholesterol Digitonide

Days	Body water	Serum cholesterol digitonide	Days	Body water	Serum cholesterol digitonide
0- 2	0.48		21-22	0.61	
2		0.011	22		0.051
4		0.015	25	0.51	
4- 5	0.70		26		0.049
6- 7	0.67		29		0.049
8		0.028	36		0.057
10-11	0.68		37	0.65	
14		0.034	43		0.054
15-16	0.64		44	0.70	
17		0.043	52		0.053

hydrogen is measured; *i.e.*, hydrogen atoms in OH , NH_2 , $CONH_2$, etc. The hydrogen atoms of the water, however, are in an overwhelming proportion.

EXPERIMENTAL

The subject was a 22 year-old lean white male in excellent health. Determinations of serum cholesterol by the Sperry-Schoenheimer method (4) shortly before and during the course of the study revealed the following: total cholesterol 175.0 mg. per 100 ml. of serum (range 158 to 192); ratio of esterified to free cholesterol 2.49 (range 2.28 to 2.89).

Deuterium was administered orally as a 10 per cent solution of D_2O . In the first 48 hours of the experiment, 4500 ml. of 10 per cent D_2O were given in ten doses of 450 ml. For the following 21 days 180 ml. of 10 per cent D_2O were given daily in single doses. The daily dosage in the final 18 days was 200 ml. of an 11.5 per cent solution.

The deuterium concentrations in body water were determined periodically by analysis of urine specimens. They ranged between 0.48 and 0.70 with an average of 0.63 atom per cent excess deuterium (Table I).

Cholesterol was isolated from the serum by precipitation as the digitonide; the water obtained on combustion of the digitonide was reduced with zinc and the deuterium concentration of the hydrogen gas evolved was determined in the mass spectrometer. The isotope analyses are presented in Table I.

The results are plotted in Fig. 1, as $\log (1 - (C_t/C_\infty))$ versus time in days. C_t is the isotope concentration at any time t ; we estimate C_∞ , the maximum isotope concentration in the digitonide, to be 0.054 atom per cent excess. $t_{\frac{1}{2}}$ as estimated from Fig. 1 is 8 days.

Plasma volume determinations were performed with the dye T-1824 and the single 10 minute point technique (5).

SUMMARY

1. The half life time of serum cholesterol in one normal man was found to be 8 days, the turnover time 12 days.
2. The total body water in a normal man was found to be 72 per cent of the body weight.

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DETERMINATION OF AMMONIA EVOLVED FROM α -AMINO ACIDS BY *peri*-NAPHTHINDAN-2,3,4-TRIONE HYDRATE AND ITS *m*-NITRO DERIVATIVE

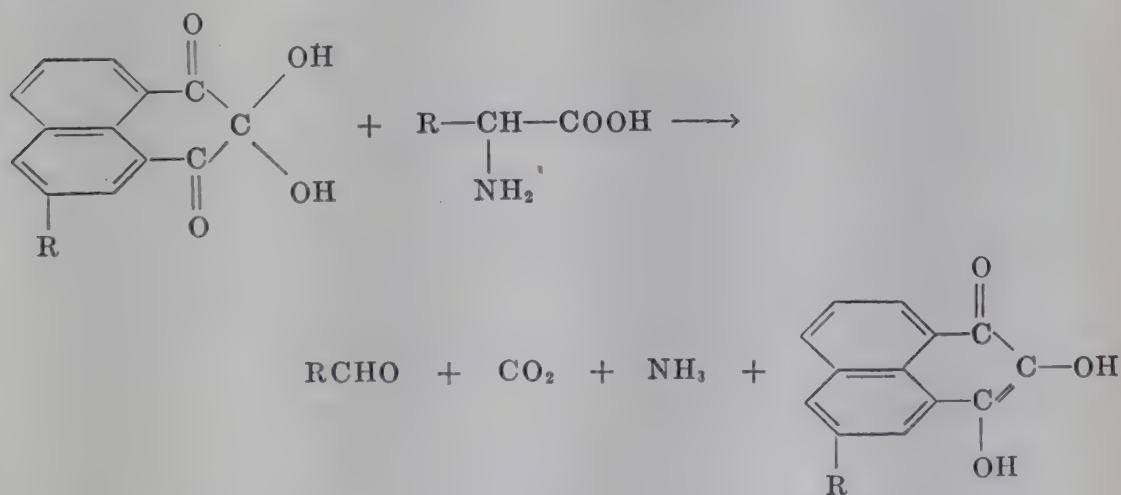
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MacFadyen (1) described a method for the estimation of α -amino acids by means of the ammonia evolved by the action of ninhydrin at pH 1.0 to 2.5. At this pH, however, the yield of ammonia from some amino acids was found to be incomplete. The amounts of amino acids used in this method ranged from 6 to 12 mg. (equivalent to 1 to 1.5 mg. of ammonia nitrogen) and the procedure described was far from simple.

peri-Naphthindantrione hydrate has been used for the estimation of α -amino acids from the aldehydes liberated (Moubasher (2), Moubasher and Awad (3)) and from the carbon dioxide evolved (Moubasher and Sina (4)).



R = H or NO₂

In the present study, we have found that when α -amino acids are treated with an excess of *peri*-naphthindan-2,3,4-trione hydrate or its *m*-nitro derivative at pH 4.7, followed by treatment with sodium hydroxide solution, ammonia is evolved quantitatively. The reaction is complete in a few minutes and the whole estimation requires about 15 minutes. Satisfactory results were obtained with 1 to 2 mg. of the α -amino acid.

The molecular structures degraded by *peri*-naphthindan-2,3,4-trione hydrate have been discussed previously (4-6). As in the case of ninhy-

drin, sarcosine and its analogues do not yield ammonia with *peri*-naphthindan-2,3,4-trione hydrate, though they give off CO_2 (4). Also, both aspartic acid and glutamic acid evolve 2 molecules of carbon dioxide by the action of *peri*-naphthindantrione hydrate (4), but yield only 1 molecule of ammonia.

EXPERIMENTAL

Reagents—

1. *peri*-Naphthindan-2,3,4-trione hydrate, freshly crystallized and powdered.
2. *m*-Nitro-*peri*-naphthindan-2,3,4-trione hydrate, freshly crystallized and powdered.
3. Buffer of pH 4.7 prepared by grinding and thoroughly mixing 17.67 gm. of sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) and 8.4 gm. of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) (7). 10 gm. were dissolved in sufficient distilled water and the solution was adjusted to 100 cc. (1 cc. of solution is equivalent to 100 mg. of buffer).
4. A standard solution of 0.01 N sulfuric acid.
5. Methyl orange solution (0.2 gm. per liter of water).
6. 40 per cent sodium hydroxide solution.
7. 4 per cent solution of boric acid (Analar).

Apparatus—

1. Rehberg micro burette of 2 cc. capacity.
2. Two micro pipettes of 1 cc. capacity, one for the buffer solution and the other for the amino acid solution.
3. Reaction and distilling apparatus (Fig. 1). It is made of Pyrex glass and consists of a steam generator (*E*) connected by means of a ground joint with the reaction vessel (*A*), to which is attached a small separating funnel (*B*). The upright condenser (*C*), the lower end of which dips under the solution in the receiver (*D*), is attached to the reaction vessel by means of a ground joint (*F*).

Procedure

1 cc. of amino acid solution (equivalent to 1 to 2 mg.) was introduced into the reaction flask *A* through the funnel *B*, followed by 0.5 cc. of the buffer solution of pH 4.7 (equivalent to 50 mg. of the solid buffer). The funnel *B* was repeatedly washed with small amounts of distilled water. Then 20 mg. of the reagent (*peri*-naphthindantrione hydrate or its *m*-nitro derivative) were introduced through the joint *F* and washed down with a small amount of water so that the total volume of solution in *A* did not exceed 10 cc. 2 cc. of distilled water or of 4 per cent boric acid (Analar) and 2 drops of methyl orange are now placed in the receiver *D*.

The condenser *C* must be well cooled. A continuous current of steam is then passed through the reaction flask *A*, which is now also heated. Heat-

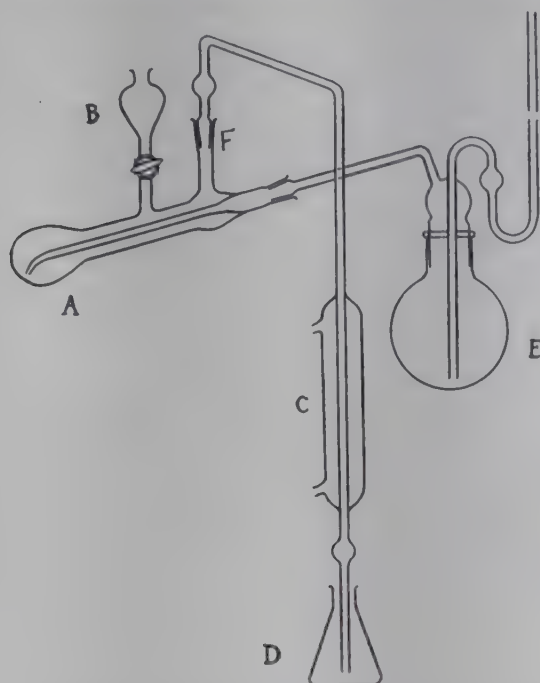


FIG. 1. Reaction and distilling apparatus

TABLE I

Reaction of Amino Acids with peri-Naphthindantrione Hydrate and its Nitro Derivative

1 mg. of the amino acid was used for each determination, except where otherwise stated.

Amino acid	<i>peri</i> -Naphthindantrione hydrate		<i>m</i> -Nitro- <i>peri</i> -naphthindantrione hydrate	
	0.01 N H ₂ SO ₄	Recovery	0.01 N H ₂ SO ₄	Recovery
	cc.	per cent	cc.	per cent
Alanine.....	1.1	98	2.28*	102
Valine.....	0.85	99	0.86	100
Leucine.....	0.79	102.5	0.75	98
Isoleucine.....	0.78	101	1.58*	103
Aspartic acid.....	0.76	101	0.76	101
Phenylalanine.....	0.62	103	0.64	107

* 2 mg. of the amino acid were used.

ing of *A* and *E* should be regulated so that only a slow stream of steam passes through the hot solution in *A*. The color of the solution in *A*, which was yellow at first, changes to orange and finally to red on heating. After boiling the solution in *A* for 10 minutes, during which time it be-

comes concentrated to about half its volume, 2 cc. of 40 per cent sodium hydroxide solution are added through the funnel *B*. The color of the solution changes to violet and finally to deep green. The solution was steamed for 5 minutes more. During the distillation, the color of the indicator in the receiver *D* was observed and the standard sulfuric acid solution (0.01 *N*) was added gradually to neutralize the liberated ammonia as soon as the yellow color appears. By performing the reaction and distillation in the same apparatus the technique is simplified and the sources of errors are minimized.

The results obtained by this method are presented in Table I.

SUMMARY

1. α -Amino acids quantitatively evolve the ammonia of their α -amino groups by the action of *peri*-naphthindan-2,3,4-trione hydrate and its *m*-nitro derivative in an aqueous medium at pH 4.7.

2. A simple and rapid submicromethod is described for the estimation of the ammonia evolved from α -amino acids.

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SERUM CATECHOLASE: PROPERTIES OF THE ENZYME AND NATURE OF AN ENDOGENOUS INHIBITOR*

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The occurrence of phenolases in plants has been known for many years (2). It has been only comparatively recently that such enzymes have been described in mammalian sources (3-5), including the histochemical demonstration of dopa oxidase activity in the leucocytes of blood (6). During an investigation of the relationship between enzymes and steroid hormones, an enzyme system was found in human blood serum which catalyzes the oxidation of catechol. Serum also contains an inhibitor, of variable concentration, which retards the oxidation of catechol during the early phases of the reaction. This report describes the properties of catecholase of human blood serum and the nature of the inhibitor. The activity of the enzyme and inhibitor in sera from various sources is described elsewhere (7).

EXPERIMENTAL

Methods

Fasting blood was drawn from the antecubital vein, and serum was separated after normal clotting had occurred. Catecholase activity of the serum was determined manometrically by measurement of oxygen uptake in the Warburg apparatus. Since the rate of catechol oxidation is markedly affected by even small changes in pH, the serum was first "neutralized" (8, 9) by removal of excess bicarbonate with hydrochloric acid. This was effected by adding serum and acid to the main chamber of Warburg vessels and alkali to the central well. The flasks, attached to lightly greased manometers, were then equilibrated in the Warburg bath at 37° for 1 hour. Most of the experiments were carried out at pH 7.4 with 1 ml. of serum. Under these conditions, generally 0.025 m.eq. of acid were required for bicarbonate neutralization.

Following the neutralization equilibration, the flasks were removed from the bath, and the following solutions were added to the main chamber: 1 ml. of 0.5 M phosphate buffer; other materials studied for their effect on

* Presented in part at a meeting of the Federation of American Societies for Experimental Biology at Detroit, April, 1949 (1).

the reaction, adjusted to the pH of the buffer; and water to bring the volume to 2.5 ml. Controls contained water in place of serum. The substrate was dissolved in 0.2 M phosphate buffer, the solution was adjusted to the pH of the contents of the main compartment, and 0.5 ml. was added to the side arm. Substrate solutions were prepared immediately before use. The flasks with attached manometers were equilibrated in the bath for 10 minutes. The stop-cocks were then closed, the zero reading was taken, and the side arm contents were tipped in. Readings were taken every 10 minutes for several hours. The flasks were then removed from

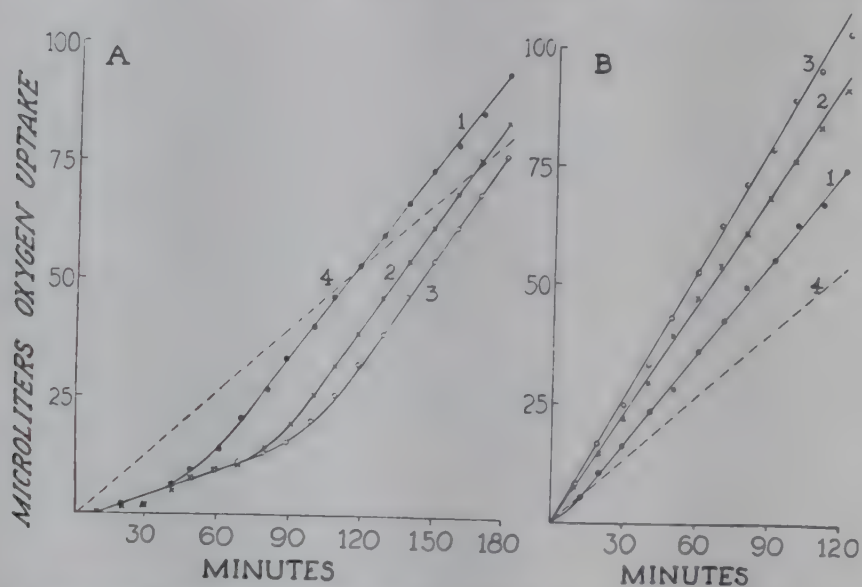


FIG. 1. Oxidation of catechol in the presence of serum. A, serum with large amount of endogenous inhibitor; B, serum with small amount of endogenous inhibitor. Serum added as follows: Curve 1, 0.5 ml.; Curve 2, 1.0 ml.; Curve 3, 1.5 ml.; Curve 4, control. The side arms contained 4 mg. of catechol. The central wells contained 0.2 ml. of 10 per cent KOH. Gas phase, air; temperature 37°; pH 7.4.

the bath, and the pH of the main chamber was checked with a glass electrode.

Results

Occurrence of Serum Catecholase

Preliminary experiments demonstrated that neither tyrosine nor *p*-cresol was oxidized in the presence of serum. When catechol was added to serum, however, oxidation occurred. There was an initial lag period during which oxidation was retarded. After an interval, which varied with the source and amount of serum used, the oxidative rate was accelerated, and soon exceeded the rate of autoxidation of catechol. The rate of oxygen uptake eventually reached a maximum and then remained constant for several hours. These relationships are brought out in Fig. 1, which shows

typical experiments on blood sera containing large and small amounts of inhibitor.

The factor in serum which catalyzes the oxidation of catechol is probably enzymatic in nature since, as described below, it satisfies several criteria for enzymatic activity, such as inactivation by heat and specificity. Of several mono- and diphenols tried as substrates, only catechol oxidation was accelerated in the presence of serum. The factor will therefore be referred to as serum catecholase. A subsequent section of this investiga-

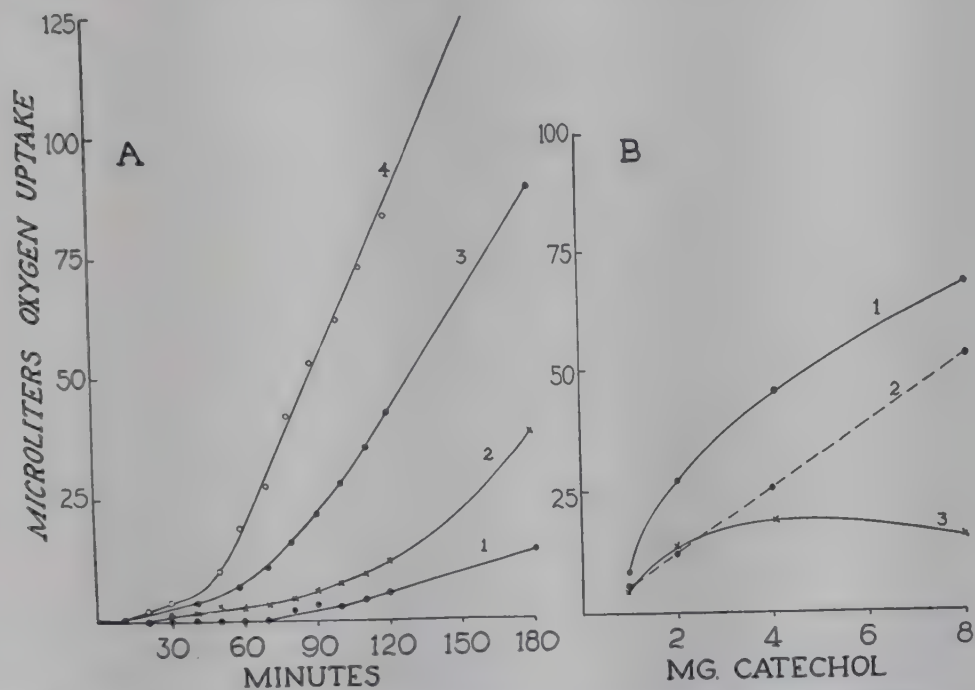


FIG. 2. Effect of catechol concentration on serum catecholase activity. A, oxidation-time curves. The experimental flasks contained 1.0 ml. of serum. The side arms contained catechol as follows: Curve 1, 1 mg.; Curve 2, 2 mg.; Curve 3, 4 mg.; Curve 4, 8 mg. Other conditions as in Fig. 1. B, oxidation-concentration curves. Oxygen uptakes during 3rd hour. Curve 1, experimental flasks; Curve 2, controls; Curve 3, net uptake in experimental flasks.

tion shows that serum catecholase inhibitor is probably a sulfhydryl compound.

Properties of Serum Catecholase

The properties of serum catecholase were investigated, for the most part, with serum showing appreciable inhibitory activity, since it was desired to determine the effect of various conditions on the inhibitor as well as on the enzyme.

Catecholase activity of serum was estimated from the difference in the rates of oxygen uptake between experimental flasks and corresponding control flasks. The inhibitory time, obtained by extending the linear

portion of the oxygen uptake curve to the time axis, was used as a measure of inhibitor activity.

Effect of Substrate Concentration—As the concentration of catechol added to serum was increased from 1 to 8 mg. per flask (Fig. 2), the inhibitory time decreased, while the rate of oxidation following the end of the lag period was augmented. Autoxidation of catechol proceeded in a linear fashion with time for all concentrations used, and the rate of oxygen uptake was proportional to the catechol concentration. Catecholase activity

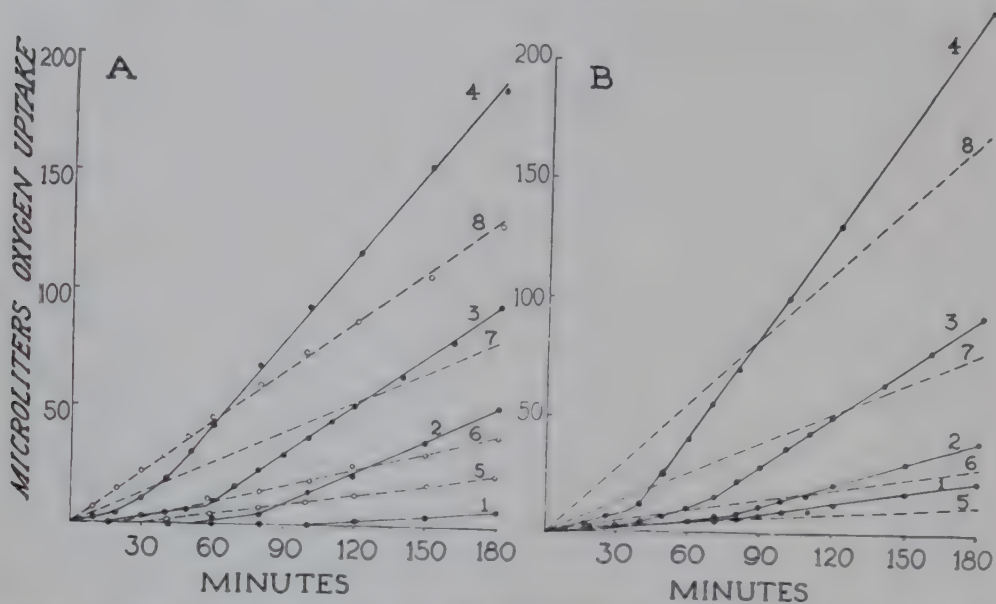


FIG. 3, A. Effect of temperature on serum catecholase activity. The experimental flasks contained 1.0 ml. of serum. Temperature of Warburg bath: Curves 1, 2, 3, 4, experimental flasks at 25°, 31°, 37°, and 43° respectively; Curves 5, 6, 7, 8, control flasks at 25°, 31°, 37°, and 43°, respectively.

FIG. 3, B. Effect of pH. The experimental flasks contained 1.0 ml. of serum, neutralized to and buffered with 0.5 M phosphate buffer at the following pH values: Curve 1, pH 6.6; Curve 2, pH 7.0; Curve 3, pH 7.4; Curve 4, pH 7.8; Curves 5, 6, 7, 8, controls at pH 6.6, 7.0, 7.4, and 7.8, respectively. Other conditions as in Fig. 1.

of serum during the 3rd hour was at a maximum when approximately 4 mg. of catechol were added.

Effect of Temperature and pH—The effects of increasing the temperature of the Warburg bath from 25° to 43° with maintenance of pH 7.4, or of increasing the pH from 6.6 to 7.8 with temperature kept constant at 37°, were similar (Fig. 3): the inhibitory time was diminished, while the maximum rate of oxidation increased. Moreover, catecholase activity during the 3rd hour increased continuously as either temperature or pH was raised, over the range of conditions studied, and no maximum activity was attained. All pH determinations were made at 25°.

Color Development—One of the characteristic properties of plant and

animal phenoloxidascs is the transformation of the substrate to a colored oxidation product (10). Serum catecholase also showed this property. No color developed during the lag period following addition of catechol to serum; however, as soon as the rate of oxygen uptake became accelerated, a pink color appeared which turned brown in a short time. The depth of color developed in 3 hours was more intense as the serum concentration, substrate concentration, temperature, or pH was raised, over the whole range of conditions observed. No appreciable color developed in control flasks.

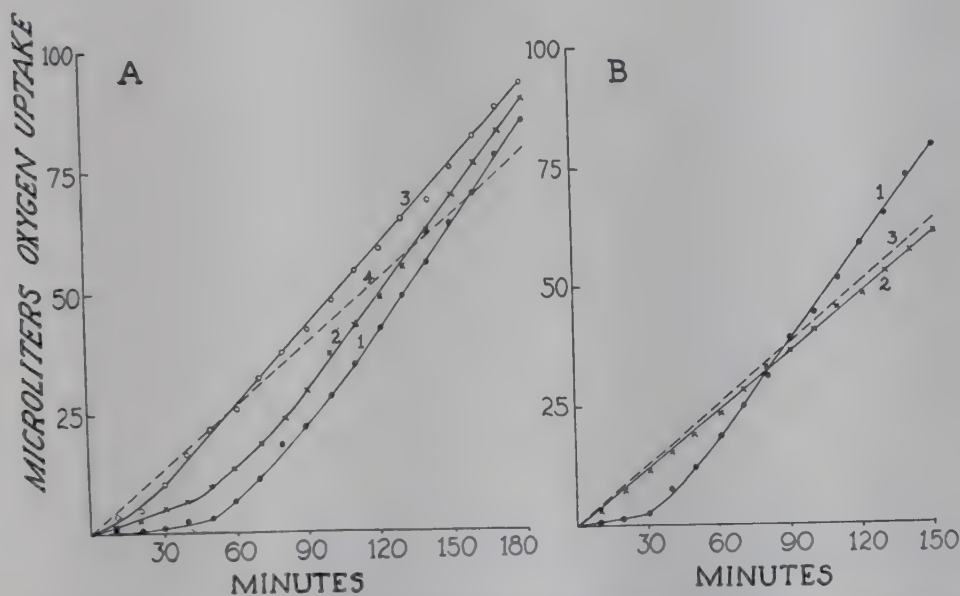


FIG. 4. Effect of heat on serum catecholase activity. A, the experimental flasks contained 1.0 ml. of serum. Curve 1, unheated serum; Curve 2, placed in water bath at 60° for 15 minutes; Curve 3, heated at 60° for 120 minutes; Curve 4, control. B, the experimental flasks contained 0.5 ml. of serum. Curve 1, unheated serum; Curve 2, placed in water bath at 80° for 15 minutes; Curve 3, control. Other conditions as in Fig. 1.

Heat Inactivation—Effect of heat on serum catecholase activity was studied by incubation of neutralized serum at 60° or 80° prior to determination of activity. No coagulation took place when 1 ml. of neutralized serum, buffered at pH 7.4 and diluted to 2.5 ml., was heated in the water bath at 60° for 2 hours. At 80°, however, coagulation occurred. When 0.5 ml. of serum was heated at 80° under the same conditions, there was no coagulation in the time required for the experiment. The results of these experiments are shown in Fig. 4. Heating of serum for 15 minutes at 60° led to the partial destruction of the inhibitor, and thus the inhibitory time was diminished. Maximum catecholase activity, on the other hand, decreased only slightly. When serum was incubated at 60° for 120 minutes,

the lag period was only transitory, while enzymatic activity, although diminished, was still present. Incubation of serum for 30 and 60 minutes at 60° produced intermediate effects on the inhibitor and on catecholase activity.

Inhibitor and enzyme were both completely inactivated when serum was maintained at a temperature of 80° for 15 minutes. Even under these conditions, however, when no enzymatic catalysis of catechol oxidation was

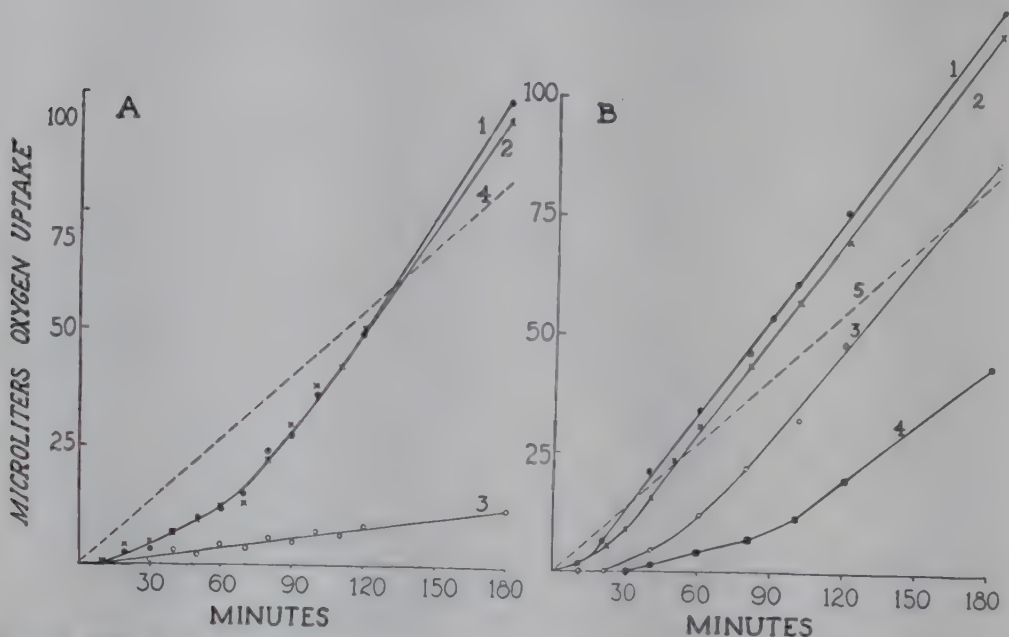


FIG. 5. Effect of cyanide on serum catecholase activity. The experimental flasks contained 1.0 ml. of serum. When cyanide was added to the main compartment the KOH in the central wells was removed following neutralization of the serum, and replaced with KCN-KOH mixtures as suggested by Umbreit (22). A, serum with large amount of endogenous inhibitor. Concentration of cyanide added as follows: Curve 1, none; Curve 2, 1×10^{-6} M; Curve 3, 1×10^{-4} M; Curve 4, controls with or without added cyanide. B, serum with small amount of endogenous inhibitor. Cyanide added as follows: Curve 1, none; Curve 2, 1×10^{-4} M; Curve 3, 1×10^{-3} M; Curve 4, 1×10^{-2} M; Curve 5, controls with or without added cyanide. Other conditions as in Fig. 1.

apparent, a brown color of diminished intensity developed in the serum-catechol solution. This observation demonstrates the caution required in the interpretation of colorimetric measurements of phenolase activity.

Effect of Cyanide—Highly purified plant phenolases are copper-containing enzymes (11, 12) which are inhibited by cyanide. Mammalian tyrosinase and dopa oxidase presumably also contain copper and are sensitive to cyanide (5). Serum catecholase activity was also affected by cyanide (Fig. 5). The effectiveness of cyanide inhibition, however, was dependent on the amount of endogenous inhibitor already present in the serum.

With serum which normally had a long lag period, a concentration of 1×10^{-4} M cyanide extended the inhibitory time for the duration of the experiment. The same concentration of cyanide, on the other hand, increased the inhibitory time only slightly with serum which had a small amount of endogenous inhibitor, while maximum catecholase activity was unaffected. With a concentration of 1×10^{-3} M cyanide, the inhibitory time was increased further, although ultimate enzymatic activity remained unchanged. When the cyanide concentration was increased to 0.01 M,

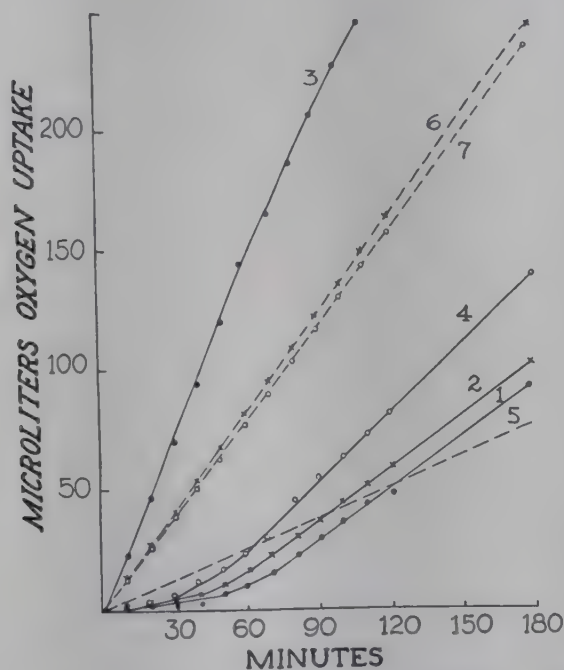


FIG. 6. Effect of metals on serum catecholase activity. The experimental flasks contained 1.0 ml. of serum. Metals added as follows: Curve 1, none; Curve 2, 1×10^{-5} M Cu^{++} ; Curve 3, 1×10^{-3} M Cu^{++} ; Curve 4, 1×10^{-3} M Fe^{+++} ; Curve 5, control without metals; Curve 6, control + 1×10^{-3} M Cu^{++} ; Curve 7, control + 1×10^{-3} M Fe^{+++} . Conditions otherwise as in Fig. 1.

however, catecholase activity disappeared, and, although the inhibitory time was again extended, the rate of oxygen uptake eventually attained the rate of autoxidation of catechol. It seems likely, therefore, that at lower concentrations cyanide acts primarily in potentiating the effect of the endogenous inhibitor, and only at higher concentrations does cyanide inhibit catecholase activity.

Cyanide, in all concentrations used, had no effect on the autoxidation of catechol.

Effect of Metals—Kubowitz (11) has shown that copper can reverse the inactivation of tyrosinase activity effected by addition of cyanide and dialysis. Fig. 6 shows the effect of copper and iron on serum catecholase

activity. Copper at 1×10^{-3} M concentration markedly accelerated the enzymatic and non-enzymatic oxidation of catechol; moreover, the inhibitory time with serum was completely abolished. When the copper concentration was decreased to 1×10^{-5} M, maximum catecholase activity remained practically unchanged, while the inhibitory time was somewhat shortened.

The effect of iron on serum catecholase activity was in sharp contrast to that of copper. Although iron at 1×10^{-3} M concentration accelerated catechol oxidation in the iron-catechol system as much as an equimolar

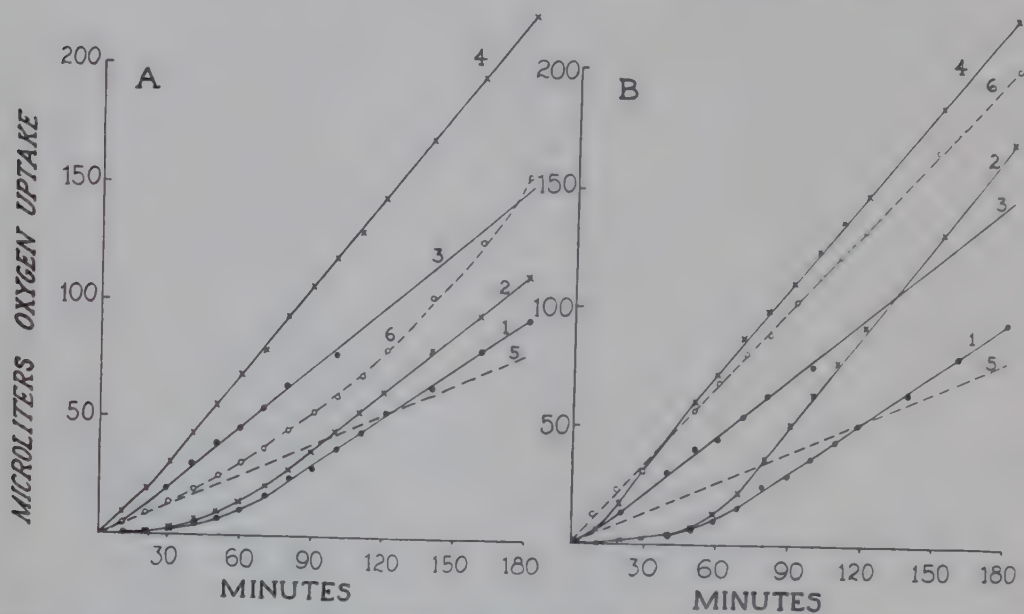


FIG. 7, A. Effect of PABA on serum catecholase activity. 1.0 ml. of serum containing large amount of endogenous inhibitor: Curve 1, without PABA; Curve 2, 0.01 M PABA. 1.0 ml. of serum containing small amount of endogenous inhibitor: Curve 3, without PABA; Curve 4, 0.01 M PABA. Curves 5 and 6, controls without and with 0.01 M PABA, respectively.

FIG. 7, B. Effect of sulfanilamide; otherwise identical to A. Other conditions as in Fig. 1.

concentration of copper, the serum-iron-catechol system showed a depressed rate of oxygen uptake. Moreover, iron had only a slight effect in decreasing the inhibitory time. It would appear, therefore, that iron added to serum is effectively bound and cannot function in accelerating catechol oxidation.

Effect of Plant Tyrosinase Inhibitors—Sulfanilamide and *p*-aminobenzoic acid (PABA) (13) and thiouracil (14) apparently inhibit plant tyrosinase activity. The effect of these compounds on serum catecholase activity was therefore determined (Figs. 7 and 8). When catechol was added to 0.01 M PABA, there was initially no increase in the rate of oxidation, nor

was there any immediate color development. After approximately 30 minutes, however, the rate of oxygen uptake became accelerated and a reddish color appeared. At the end of the 3rd hour the effect of PABA on catechol oxidation was very marked and a strong brown color had developed. The same concentration of PABA, added to serum containing appreciable endogenous inhibitor, had no effect on the inhibitory time, while the accelerative phase of catechol oxidation was retarded. When added to serum which had a short lag period, PABA inhibited the apparent enzymatic activity. Thus catecholase activity during the 3rd

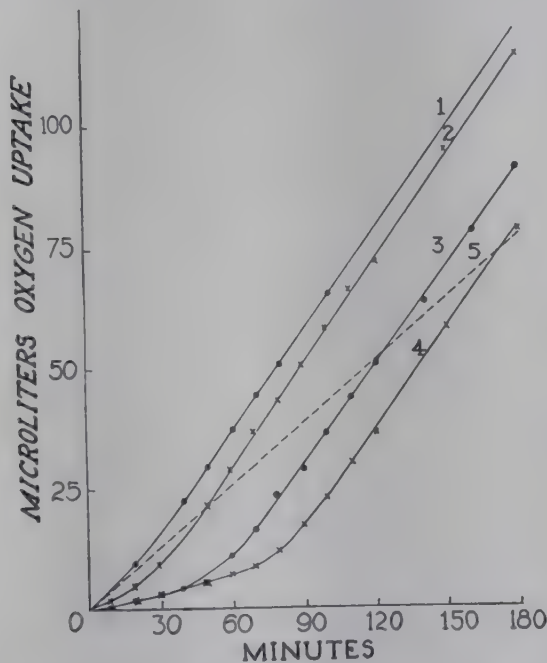


FIG. 8. Effect of thiouracil on serum catecholase. Curves 1 and 2, serum with small amount of endogenous inhibitor; Curve 1, without thiouracil; Curve 2, 1×10^{-4} M thiouracil. Curves 3 and 4, serum with large amount of endogenous inhibitor; Curve 3, without thiouracil; Curve 4, 1×10^{-4} M thiouracil. Curve 5, control without or with 1×10^{-4} M thiouracil. Conditions otherwise as in Fig. 1.

hour without added PABA was equivalent to 23.5 μ l. of oxygen uptake, while the addition of PABA reduced the activity to 9.5 μ l. In the concentration used, therefore, PABA has no effect on endogenous catecholase inhibitor, but apparently either inhibits serum catecholase activity itself or else is bound by other components of serum and prevented from exerting its accelerative effect on catechol oxidation.

Sulfanilamide had an effect somewhat different on catechol oxidation than PABA. The addition of catechol to 0.01 M sulfanilamide was followed by an immediate increase in the rate of oxidation, and a pink color developed rapidly. When added to serum, sulfanilamide had practically no

effect on the inhibitory time, while apparent maximum catecholase activity was decreased.

Fig. 8 shows that the only effect of 1×10^{-4} M thiouracil was to increase the inhibitory time. There was no inhibition of maximum enzymatic activity. Thiouracil at a concentration of 1×10^{-4} M had no effect on the autoxidation of catechol. When the thiouracil concentration was increased to 1×10^{-3} M, however, both the enzymatic oxidation and the autoxidation of catechol were inhibited.

Specificity of Serum Catecholase—In addition to catechol, the *p*-diphenols, hydroquinone and homogenistic acid,¹ and the *o*-diphenols, epinephrine

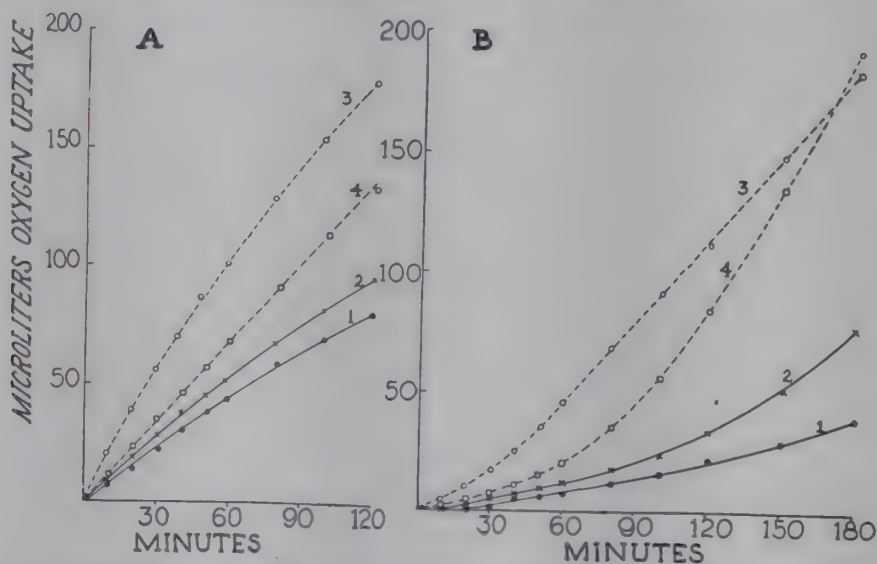


FIG. 9. Specificity of serum catecholase. The substrates were added in concentrations equimolar to 4 mg. of catechol. The experimental flasks contained 1.0 ml. of serum. A, Curve 1, homogenistic acid; Curve 2, hydroquinone; Curve 3, homogenistic acid control; Curve 4, hydroquinone control. B, Curve 1, dopa; Curve 2, epinephrine; Curve 3, dopa control; Curve 4, epinephrine control. Other conditions as in Fig. 1.

and dopa, were tried as substrates for serum catecholase (Fig. 9). With none of these substrates did the rate of oxidation in serum attain the rate of autoxidation. Moreover, neither tyrosine nor *p*-cresol was oxidized in the presence of serum over a period of several hours, even following the addition of small amounts of catechol. It would appear, therefore, that under the conditions specified serum phenolase activity is specific for catechol.

Nature of Endogenous Inhibitor

There were several indications, in the experiments described above, that the endogenous inhibitor in serum which delayed oxidation of catechol was

¹ The author is indebted to Dr. Howard B. Lewis for lead homogenistic acid, and to Dr. Arthur Furst for isolation of homogenistic acid and for the iodoacetate used.

a sulfhydryl compound. Copper, for example, catalyzes the oxidation of reduced glutathione, while iron is ineffective in this regard (15, 16). Moreover, cyanide inhibits almost completely the oxidation of sulfhydryl groups by various oxidizing agents (17). Figge (18) has shown that glutathione inhibits melanin formation in tyrosine-tyrosinase mixtures; and more recently it was found (19, 20) that human skin contains a dialyzable sulfhydryl compound which inhibited the autoxidation of dopa and the catalytic oxidation of tyrosine by plant tyrosinase. The inhibition could be eliminated by the addition of copper or of the sulfhydryl reagents, iodoacetamide and *p*-chloromercuribenzoic acid. The effect of several sulfhydryl reagents on the endogenous inhibitor in serum was therefore determined.

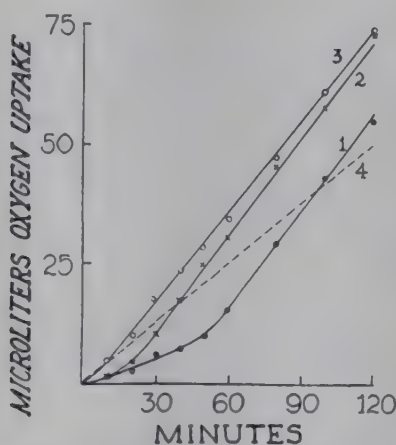


FIG. 10. Effect of *p*-chloromercuribenzoic acid on serum catecholase activity. The experimental flasks contained 1.0 ml. of serum. *p*-Chloromercuribenzoate added as follows: Curve 1, none; Curve 2, 1×10^{-4} M; Curve 3, 1×10^{-3} M; Curve 4, controls without or with added mercuribenzoate. Conditions otherwise as in Fig. 1.

Iodoacetate,¹ in a concentration of 1×10^{-3} M, had no effect on the lag period of catechol oxidation. When 4×10^{-3} M tetrathionate was added to serum, the inhibitory time was approximately halved. The most marked effect, however, was obtained with *p*-chloromercuribenzoic acid (Fig. 10). The inhibitory period was almost completely abolished by 1×10^{-3} M and considerably reduced by 1×10^{-4} M mercuribenzoate. None of the sulfhydryl reagents used materially affected maximum catecholase activity or influenced the autoxidation of catechol.

Serum dialyzed against 0.9 per cent saline showed no change in either inhibitory time or maximum catecholase activity over undialyzed serum, while mercuribenzoate was equally effective in abolishing the lag period of dialyzed serum. It appears likely, therefore, that the substance in blood serum which initially inhibits catechol oxidation is a non-dialyzable sulfhydryl material.

The release of catecholase inhibition in blood serum probably involves the oxidation of the inhibitory sulfhydryl groups. This is indicated by

the accelerative effect of copper on the release of inhibition and on the prolongation of inhibition with cyanide. Catechol is apparently necessary for the sulfhydryl oxidation, since neither keeping the serum refrigerated for several days before determination of catecholase activity nor increasing the time of the neutralization equilibration materially decreases the inhibitory time. The mechanism of oxidation possibly involves the initial oxidation of catechol to *o*-benzoquinone, which then oxidizes the sulfhydryl groups and is in turn reduced to catechol again, until all of the sulfhydryl groups are oxidized. Miller and Dawson (21) have utilized a similar reaction in their chronometric method for measurement of catecholase activity, using ascorbic acid as the reducing agent.

SUMMARY

1. Human blood serum contains an enzyme which accelerates the oxidation of catechol, but not tyrosine, *p*-cresol, hydroquinone, homogentisic acid, epinephrine, or dopa; the system is referred to as serum catecholase.

2. Serum also contains an inhibitor, of variable concentration, which initially retards the oxidation of catechol. The inhibitor has the characteristics of a non-dialyzable, sulfhydryl material.

3. Serum catecholase activity is accelerated and the inhibitory time decreased by increases in temperature from 25° to 43° and by increases in pH from 6.6 to 7.8.

4. Heat of 80° inactivates both the enzyme and the inhibitor.

5. Cyanide inhibits catecholase activity and prolongs the effect of the endogenous inhibitor.

6. Copper abolishes the lag period in the oxidation of catechol by serum and enhances catecholase activity.

7. Sulfanilamide and *p*-aminobenzoic acid have no effect on the endogenous inhibitor, but themselves inhibit apparent catecholase activity. Thiouracil prolongs the inhibitory time but has no effect on maximum enzymatic activity.

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SOME FACTORS AFFECTING THE ACTION OF 2,4-DINITRO-PHENOL ON THE OXYGEN UPTAKE OF EXCISED RAT BRAIN*

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It is well known that oxygen consumption of various types of tissue is accelerated by low concentrations of 2,4-dinitrophenol (DNP) and inhibited by only slightly higher concentrations (1-6). It is equally well known that those concentrations that augment respiration produce a reversible block to cell division (4, 7) and inhibit assimilation or synthetic processes without affecting catabolism (8-12). It would appear, then, that the exact identification of the metabolic steps or particulate components affected by this substance would constitute a contribution to our understanding of some of the chemical and functional processes of the cell. To this end it has been reported that in frog muscle DNP accelerates the hydrolysis of phosphocreatine and adenosine triphosphate (13), interferes with the phosphate uptake of respiring yeast (14), and produces, in rabbit kidney, a reversible dissociation of respiratory hydrogen transfer from phosphorylation ((15, 16); see also (17)). Cellular structure, a factor in the capacity of the brain (18) and liver (19) to oxidize carbohydrate, has also been suspected to be essential for the action of DNP (5, 6, 13, 20), particularly for the augmenting phase of action (20).

It has been shown that this ability of the brain (to respond with augmentation to DNP) is destroyed by water homogenization (20) and is not restored by reinforcing such preparations with the known cofactors for glycolysis and glucose oxidation (6, 20). In fact, the oxygen uptakes of such suspensions are found to be inhibited by concentrations of DNP that produce acceleration in slices or minces (20).

This report deals with the action of DNP on the oxygen uptake of excised rat brain as it is affected by: (1) graded concentrations of the drug, (2) the age of the animal, and (3) the type of substrate used in the respiring media.

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EXPERIMENTAL

The usual Warburg procedures to determine oxygen uptake were used. The medium was Ringer-phosphate plus MgCl_2 at a pH of 7.3. The final concentration of substrates was 1.8 mg. per ml. Only that portion of the brain known as the pallium was used (*i.e.*, the cerebral hemispheres, exclusive of the caudatum and the rhinencephalon). In those experiments in which the brain tissue was minced it was prepared in a manner previously described (21), and 100 mg., wet weight, were added to each of several vessels containing 3.0 ml. of medium. Here the gas phase was oxygen and the vessels were gassed at room temperature. In the experiments in which homogenates were used they were blended for 1 to 2 minutes, or until smooth suspensions were obtained, in an all-glass hand homogenizer (22) immersed in an ice bath. The homogenizing medium was Ringer-phosphate plus the substrate. 1 ml. of the homogenate, containing 100 mg. of brain, was then added to the main compartment of each of several vessels containing 1.0 ml. of medium. The vessels were prepared so that on the addition of the homogenate the desired concentration of DNP was obtained. In these studies the final volume was 2.0 ml. and the gas phase was air. Controls, of course, were run simultaneously on samples of the same brain respiring in media without DNP.

The rats were killed by decapitation and the brains were quickly removed and prepared so that all vessels were in the bath (38°) 20 to 30 minutes after killing. After 20 minutes of equilibration, readings were taken every half hour. Oxygen uptake in all instances was calculated as c.mm. per 100 mg. (wet weight) of brain tissue per hour. In comparative studies, particularly when rats of different ages are used, this calculation is more desirable than the conventional Q_{O_2} values. Dry weight values, although correcting for the greater water content of the new-born, fail to take into account the large amount of poorly respiring myelin present in the adult brain (21). The effect of DNP is expressed as the per cent change from control values taken simultaneously. The results are the arithmetical means of a sufficient number of experiments to give reliability to the differences found. Individual runs were, for the most part, in duplicate and, in many instances in triplicate. Agreement among them was satisfactory. 52 female adult rats and twenty new-born litters were used in these experiments.

Results

Concentration-Action Curve—Fig. 1 shows the effect of graded concentrations of DNP on the oxygen uptake of the minced adult brain as determined at half hour intervals for a 2 hour period. It can be seen that the greatest increase in oxygen uptake occurs during the first $\frac{1}{2}$ hour and with

5.0×10^{-5} M DNP. This is in agreement with the findings of others (6). However, following the changes in oxygen uptake over the 2 hour period

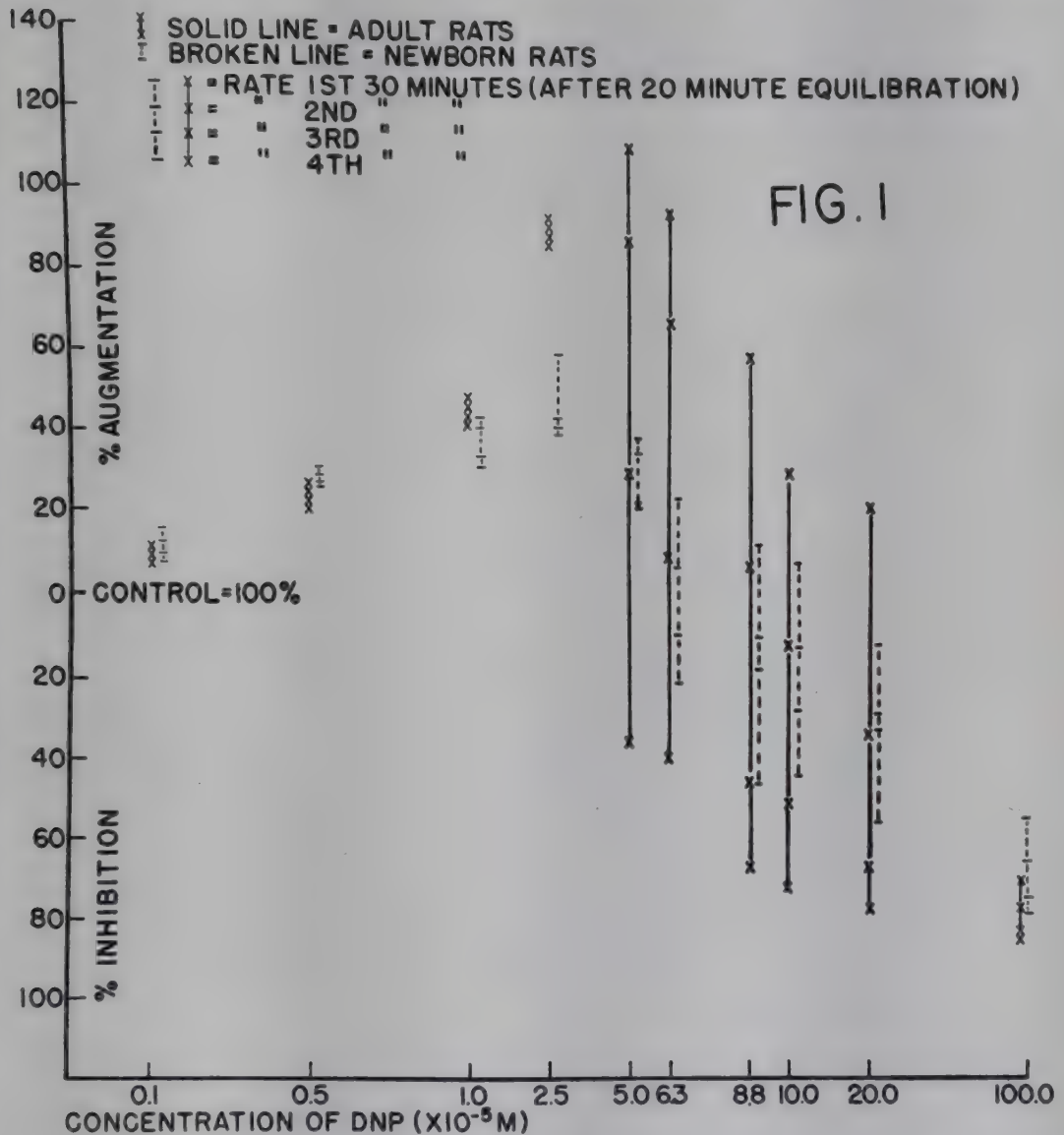


FIG. 1. Effect of graded concentrations of DNP on the oxygen uptake of minced adult rat brain. The length of each vertical line represents a continuous 2 hour period of observation with values for consecutive half hour intervals indicated by reference marks commencing at the top of each line. Control readings for each half hour are expressed as 100 per cent and form the 0 line. Data obtained on minced brain in 3.0 ml. of Ringer-glucose-phosphate solution, pH 7.3, gas phase oxygen, and bath temperature 38° .

reveals some additional information on the mode of action of DNP that is not indicated on curves obtained with single readings. Concentrations of DNP of from 1.0×10^{-6} M to 2.5×10^{-5} M result in only an accelerated oxygen uptake. This augmented rate is maintained at a fairly constant

level for the entire 2 hour period, and there is no marked tendency for the respiration to fall off, despite the fact that with 2.5×10^{-5} M concentrations the oxygen uptake of the adult brain has almost approached the maximum rate obtained in these experiments. Increasing the concentration to 5.0×10^{-5} M results in only a slight further increase in oxygen uptake during the 1st hour, but with this concentration respiration falls off very rapidly and during the 2nd hour is below that of the controls. Further increases of DNP concentration result in less augmentation and more inhibition, until finally a concentration of 5.0×10^{-4} M and above produces only inhibition.

It can be seen that concentration-action curves based on the oxygen consumption of the 1st hour would not accurately detect the onset of the inhibitory action. With such curves the first signs of inhibition would appear at about 5.0×10^{-4} M. Fig. 1 reveals that inhibition first occurs at a concentration one-tenth as great and its onset is not a gradual but a sudden one.

The respiration of excised brain tissue falls off with time. The mean oxygen uptake of the control vessels for the four consecutive half hour periods is 168, 166, 163, and 160 c.mm. per 100 mg. per hour. In Fig. 1 these values, for the adult brain, are given as 100 per cent.

Effect of Age on Concentration-Action Curves—In Fig. 1 the effect of similar concentrations of DNP on the *minced* pallium of the new-born rat is shown. It should first be pointed out that at this age low concentrations (up to 2.5×10^{-5} M) produce irregular results of a minor nature. For instance, in some runs the values found on the 2nd and even 3rd half hour intervals are higher than those obtained during the first half hour (increasing augmentation). However, as with the adult at this concentration range, there is no marked tendency for the oxygen uptake in the vessels containing DNP to fall off.

The concentration producing the greatest increase in oxygen uptake is found to be lower for the new-born brain, 2.5×10^{-5} M. Also, for most concentrations, the percentage change produced is less for the new-born than for the adult. On the other hand, the concentration producing the first signs of inhibition in the new-born is slightly higher than that required for the adult. As with augmentation, the percentage inhibition produced, for a given concentration, is less in the new-born until a concentration of 1.0×10^{-3} M is used. At this concentration the brain of both the new-born and adult is inhibited by approximately the same percentage.

The mean oxygen uptake for the control vessels containing pallium of new-born rats is 77, 76, 69, and 67 c.mm. per 100 mg. per hour for each of the four consecutive half hour periods. These values are also expressed as 100 per cent in Fig. 1.

Effect of Different Substrates—The results shown in Fig. 2 were obtained with homogenates of adult brain prepared in Ringer-phosphate solution in

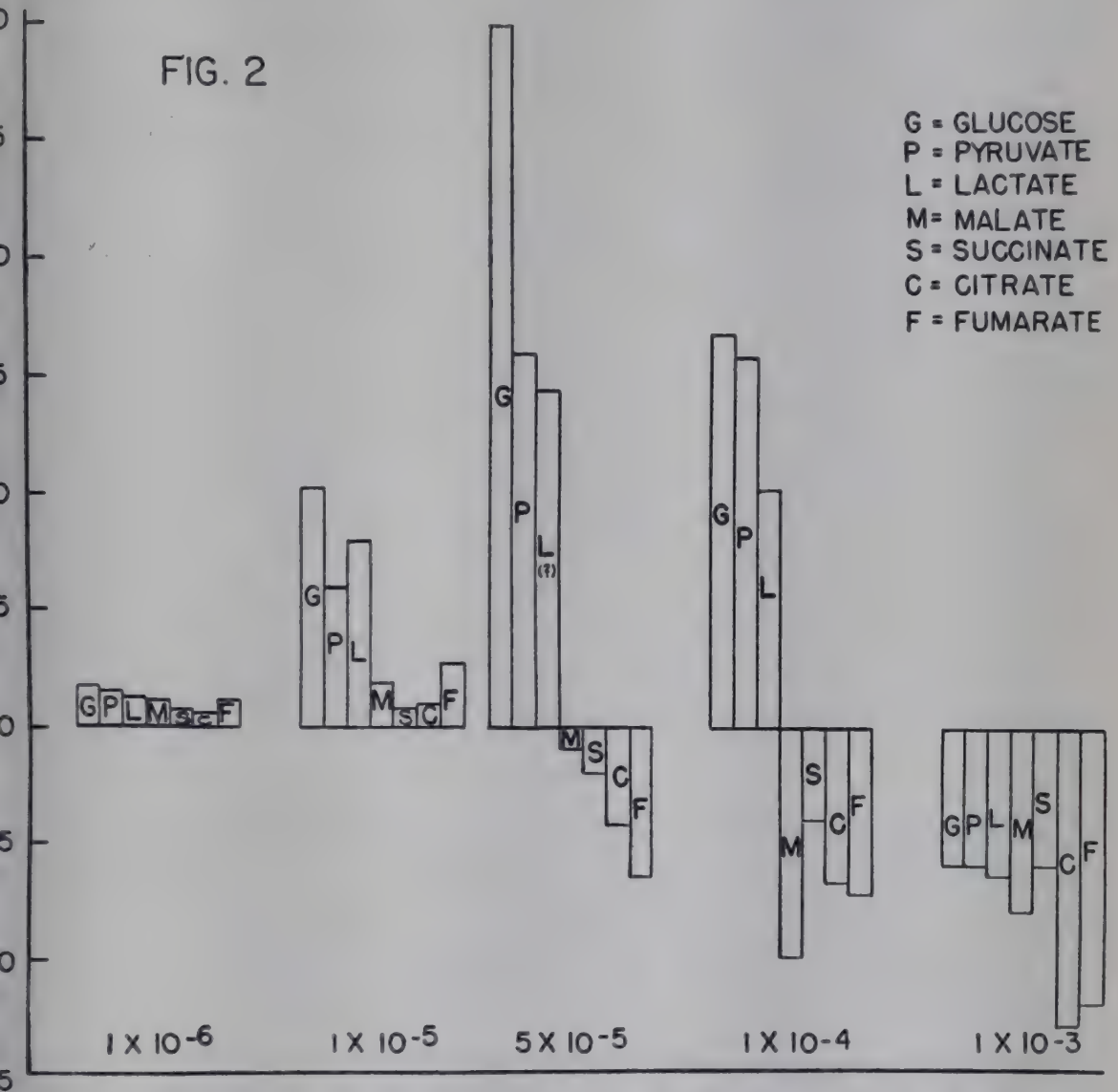


FIG. 2. Data obtained on homogenates of adult rat brain. The values are based on the oxygen consumption during the 1st hour, following 20 minutes of equilibration. Control values for the various substrates are expressed as 100 per cent and form the zero line. Changes produced by various concentrations of DNP with a given substrate are indicated by the length and direction of the column. The value for lactate at 5.0×10^{-5} M is estimated from values obtained at 6.5×10^{-5} M. Gas phase air; bath temperature 38° . Ordinate, per cent change, abscissa, concentration of DNP.

which glucose was substituted by one or the other of the substrates. The values are based on oxygen uptake during the 1st hour. Pyruvate was freshly prepared from doubly distilled acid (3 to 5 mm. of Hg), diluted,

and slowly (in an ice bath) brought to a pH 6 to 7 with 0.4 M NaOH. The other substrates were prepared from commercial preparations and used as the sodium salts. It can be seen that when either lactate or pyruvate is used as the substrate the response to various concentrations of DNP is somewhat similar to that found with glucose. The percentage change is lower, particularly with 5.0×10^{-5} M concentrations. On the other hand, when citrate, malate, succinate, or fumarate is used, only inhibition occurs with concentrations of 5.0×10^{-5} M and above. With the very low concentrations, 1.0×10^{-6} M and 1.0×10^{-5} M, there are some indications of augmentation, but it is highly probable that the slight increase found at these concentrations may be due to lactate that is usually formed during the preparation of the brain (23). Two experiments in which hexose diphosphate (HDP) was used and one with the Cori ester produced essentially the same results as those found with the above members of the citric acid cycle, no augmentation, only inhibition with DNP.

That the failure of the brain to respond to suitable concentrations of DNP with an increase in oxygen uptake when the above substrates were employed was not related to the inability to oxidize them is indicated by the oxygen uptakes in the control vessels. These were for glucose 101, pyruvate 129, lactate 122, malate 86, succinate 214, citrate 82, fumarate 88, Cori ester 115, and HDP 72. Since in these experiments homogenates were used, penetration is probably not a factor.

DISCUSSION

From the concentration-action curves shown here, three effects are seen: (1) low concentrations (up to 2.5×10^{-5} M) that produce only augmentation; (2) intermediate concentrations that first augment and then depress; (3) high concentrations that produce only inhibition. These findings, together with the observation that the inhibitory phase of action sets in suddenly at 5.0×10^{-5} M (contrasted with its absence at 2.5×10^{-5} M), suggest the probability that DNP is not limited to only one site or mode of action. The indications are that augmentation and inhibition are due to different mechanisms, each having distinctly different threshold concentrations. Under the conditions of these experiments, and for the adult female rat, the threshold concentration for the augmentation phase is about 1×10^{-6} M; for the inhibitory phase, it is 50-fold higher. It is possible that this latter value might be somewhat lower but is masked by the augmentation. However, the absence of any marked deterioration of oxygen uptake at 2.5×10^{-5} M seems to indicate that the value found is not far off. The present data do not permit any conclusions as to what mechanisms are involved in these actions.

The results obtained on the brain of the new-born rat show that the

response to the augmenting or inhibiting concentrations of DNP (from 2.5×10^{-5} M upward) is less than that found for the adult. The evidence also suggests that at this age a higher concentration of DNP is required to produce the first signs of inhibition. This latter finding does not necessarily mean that the threshold concentration for inhibition in the brain of the new-born is higher, for the curve, particularly the flat intermediate zone, indicates that considerable overlapping of augmentation and inhibition may be operating to mask the true threshold concentration.

When lactate or pyruvate is used as substrate for adult brain homogenates, the response to graded concentrations of DNP is similar in direction to that found with glucose, although the absolute increase found is less. On the other hand, it is evident that when either succinate, fumarate, malate, citrate, hexose diphosphate, or the Cori ester is substituted for glucose the augmenting phase of action of DNP is lost. In fact, those concentrations of DNP that accelerate O_2 uptake in a glucose medium produce only inhibition under such substrate conditions.

Loomis and Lipmann (24) report that when pyruvate is used as a substrate the oxygen uptake of an R_3 suspension of rabbit kidney (16) is depressed by 10^{-5} DNP. At this concentration we find that the oxygen uptake of adult rat brain is augmented by about 80 per cent during the 1st hour. The reasons for these conflicting observations undoubtedly rest on the differences in sensitivity of brain and kidney to DNP and probably on the manner in which the tissue was prepared. Bearing on the first possibility is the observation of Fuhrman and Field (25) that, at 37.5° , 3.35×10^{-5} M DNP stimulates the oxygen uptake of rat brain cortex by about 50 per cent, while under the same conditions of temperature and inhibitor concentration, kidney cortex shows a slight inhibition. At 25° , the temperature used in the experiments of Loomis and Lipmann, rat kidney cortex is augmented by about 33 per cent and brain by 77 per cent. Similar organ differences may well exist for the rabbit and it would appear likely that the concentration of DNP used by Loomis and Lipmann (24) would be in that zone where the activity on oxygen uptake is predominantly inhibitory. It is also possible that the method of preparation of the tissue is a factor, as it has been shown that this plays an important rôle in the type of response obtained with DNP (20). Dicarboxylic acid is reported to overcome the inhibition produced by DNP on an R_3 kidney suspension with pyruvate as a substrate (24), and a sufficient concentration of these acids might be present in the whole brain homogenates used here. This explanation appears unlikely, since DNP does not depress the oxygen consumption of kidney with malate or fumarate as substrate,¹ while it does, as reported here, on rat brain.

¹ Lipmann, F., personal communication (1949).

SUMMARY

1. The changes in oxygen uptake with time as produced by graded concentrations suggest that dinitrophenol has at least two different sites of action, each having distinctly different threshold concentrations. That required for augmentation is approximately 1×10^{-6} M; for inhibition the threshold concentration is 50-fold higher.

2. Concentration-action curves obtained from the brain of the newborn rat exhibit a flatter response to dinitrophenol.

3. When lactate or pyruvate is used as a substrate, the response to graded concentrations of dinitrophenol is similar in direction to that found with glucose. On the other hand, the augmentation phase of action is absent when fumarate, citrate, malate, succinate, hexose diphosphate, or the Cori ester is used as a substrate.

I am indebted to my wife, Matilda Tyler, for technical assistance in this work.

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THE OXIDATION OF PALMITIC ACID-1-C¹⁴ BY EXTRAHEPATIC TISSUES OF THE DOG*

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It is generally agreed that the liver is the principal site for the oxidation of higher fatty acids. The question as to whether extrahepatic tissues can oxidize higher fatty acids directly has been long debated. In 1942, Gemmill (1) pointed out that there was no experimental evidence to indicate the direct utilization of fat by mammalian muscle, but this view was not shared by Stadie (2) when he reviewed the subject in 1945. It remained for Lehninger (3), however, to provide the first *in vitro* evidence of the direct oxidation of a long chain fatty acid by an extrahepatic tissue. This investigator demonstrated that a rat heart muscle suspension can oxidize octanoate, laurate, and palmitate. Kidney and skeletal muscle preparations also gave indications of ability to oxidize higher fatty acids (3). Grafflin and Green have shown that kidney enzyme preparations oxidize rapidly all of the saturated, straight chain, monocarboxylic fatty acids from acetic through *n*-tridecanoic, with the exception of propionic acid. Myristic, palmitic, stearic, and oleic acids were not oxidized by the kidney system (4). Oxidation of octanoate and trilaurin by various extrahepatic tissue slices has been confirmed by Geyer *et al.* (5). The *in vivo* significance of these findings has not, as yet, been assessed. Nor has indisputable proof that oxidation of naturally occurring fats takes place in the extrahepatic tissues of the intact animal been provided.

In the present investigation, we undertook a study of the extent to which the extrahepatic tissues of the intact animal participate in the oxidation of a physiologically occurring fatty acid. Palmitic acid labeled with C¹⁴ at the carboxyl position was used, and its conversion to C¹⁴O₂ compared in normal and hepatectomized dogs.

* Aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

EXPERIMENTAL

Treatment of Dogs

For 2 weeks before study, healthy female dogs (8 to 10 kilos) were maintained on a stock diet consisting of lean meat, sucrose, bone ash, and Cowgill's salt mixture (6). Vitamin supplements were also fed daily.

Procedure for Hepatectomy—The method used here for complete excision of the liver was a modification of the two-stage operation described by Markowitz and Soskin (7). The dogs were first injected intravenously with 0.45 mg. of atropine sulfate to minimize salivation. They were then anesthetized by an intravenous injection of 35 mg. of sodium pentobarbital (nembutal) per kilo of body weight. In the first operation, the portal vein and the vena cava caudal to the liver were partially ligated. The constriction of the vessels was sufficient to induce slight cyanosis of the intestines. For the first 2 days after this operation, the animals were fed a diet consisting of milk, sucrose, and vitamin supplements. Increasing amounts of meat were fed after the 2nd day, and by the 6th day the feeding of the full amount of the meat-sucrose diet described above was resumed.

After a period varying from 2 to 4 weeks, the animals were subjected to the second stage of the hepatectomy procedure. This time anesthesia was induced by ether. Atropine sulfate was again administered intravenously. The portal vein, hepatic artery, and other structures of the hepatoduodenal ligament were separately ligated and divided. The inferior vena cava was then ligated cranial and caudal to the liver, and the liver was removed piecemeal. 5 per cent glucose was administered by intravenous drip at the rate of 60 cc. per hour after removal of the liver.

Both normal and hepatectomized dogs were kept under anesthesia for the duration of the experiment. The normal dogs received an initial injection of 35 mg. of sodium pentobarbital per kilo of body weight, and supplementary injections as needed. The hepatectomized dogs were kept under ether anesthesia with additional amounts of ether as needed.

CO₂ Collection—Collection of expired air was accomplished by tracheal cannulation. The expired air was collected in large neoprene balloons (40 inches in diameter) which acted as temporary reservoirs, and then, by the aid of suction, passed through columns of an NaOH solution. 600 m.eq. of NaOH were used per hour of collection. The NaOH-NaHCO₃ solution so formed was brought to a convenient volume and stored for analyses.

Blood Collection—Whole blood was withdrawn from the femoral artery into a heparinized syringe. A known volume of plasma (usually 3 to 5

cc.) was transferred into 30 volumes of 95 per cent ethyl alcohol, and thoroughly mixed.

Preparation of Tripalmitin Emulsion Containing C^{14} -Labeled Palmitic Acid

The emulsion contained 2 per cent tripalmitin, 2 per cent olive oil, 1 per cent glycerol monostearate, 5 per cent glucose, and 90 per cent distilled water. The tripalmitin was synthesized from palmitic acid labeled with C^{14} at the carboxyl position (8).

The weighed quantities of the tripalmitin, olive oil, and monostearate were placed in a small stainless steel Waring blender, and melted on a steam bath. Glucose in distilled water was then added. Care was taken to insure that the fat was in liquid state before its emulsification. The mixture was homogenized for 10 to 12 minutes. Temperatures of about 80° were attained in this process. The emulsion was allowed to cool, and was injected immediately after it reached body temperature ($35-37^{\circ}$). Maximal particle size, as revealed by microscopic examination, was usually less than $1\ \mu$ and never more than $3\ \mu$.

The emulsion was injected into the jugular vein, which had been previously exposed. Each dog received 27 to 30 cc.; the injection of these amounts required 4 to 5 minutes.

Methods of Analyses

CO₂ Determinations—The methods used for the determination of the total CO₂ and C^{14} content of the NaOH-NaHCO₃ solutions have been described elsewhere (9).

Lipide Analysis—The determination of the C^{14} content of the non-phospholipide fraction of plasma fatty acids is described in the next paper (10).

Results

Rates of Disappearance of Injected Palmitic Acid- C^{14} from Blood Stream of Normal and Hepatectomized Dogs

Four dogs were studied, two normal and two hepatectomized. At various intervals after the injection of the labeled tripalmitin, samples of blood were removed from the femoral artery. The C^{14} content of the plasma non-phospholipide fatty acids are recorded in Fig. 1. The values are here expressed as percentages of the injected C^{14} per 100 cc. of plasma. The removal of the injected palmitic acid- C^{14} from the blood stream was more rapid in the normal than in the hepatectomized dog. If it is assumed that each dog had a plasma volume of about 500 cc., then it becomes apparent from Fig. 1 that, in the first 30 minutes, 80 and 90 per cent respectively of the injected palmitic acid- C^{14} disappeared from the blood streams

of the normal dogs. In this same time interval 10 and 30 per cent, respectively, left the blood streams of the two hepatectomized dogs. At the end of 3 hours, the injected C¹⁴ had practically disappeared from the blood streams of the normal dogs, whereas 20 and 50 per cent, respectively, were still present in those of the hepatectomized dogs.

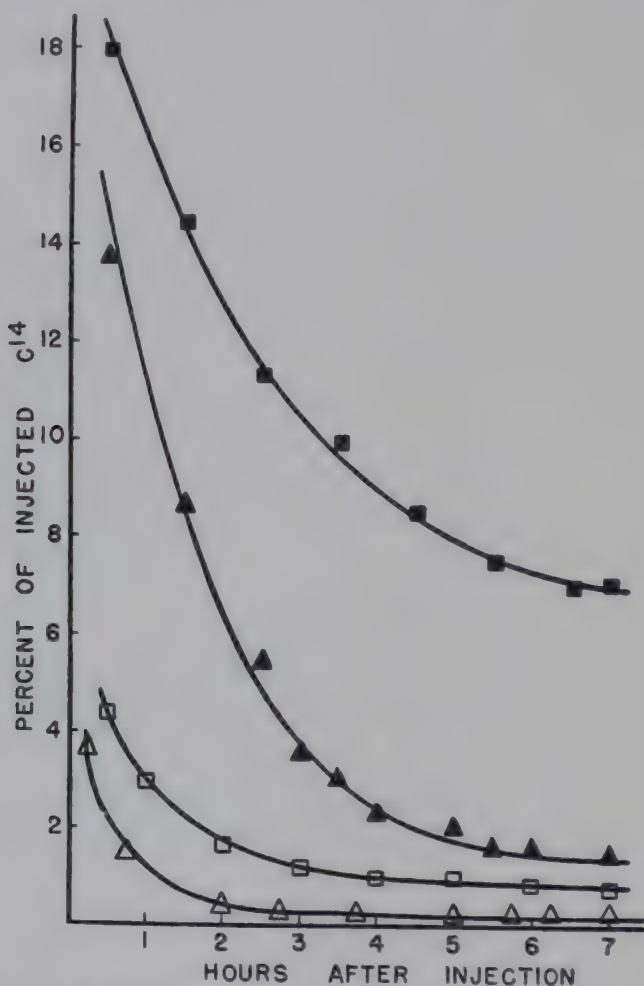


FIG. 1. Recovery of C¹⁴ in plasma non-phospholipide fatty acids of normal and hepatectomized dogs following injection of tripalmitin containing palmitic acid-1-C¹⁴. The ordinate represents per cent of injected C¹⁴ recovered in non-phospholipide fatty acids per 100 cc. of plasma. □ normal Dog C, △ normal Dog F, ▲ hepatectomized Dog B, and ■ hepatectomized Dog C.

Tangents were drawn at various points along the curves shown in Fig. 1, and their slopes were determined. The values so obtained, which are the rates of removal of the injected palmitic acid-C¹⁴, were plotted against time on semilogarithmic paper in Fig. 2. The half times of the curves for the two normal dogs were in good agreement, namely 0.65 and 0.70 hours. It is clear from Fig. 2 that, in the normal dogs, the labeled fatty acid was removed from the circulation at an exponentially decreasing rate.

In the case of the hepatectomized dogs, two distinct components appeared in the rate curves for the disappearance of the palmitic acid- C^{14} , an initial, slow component with half times of 1.5 and 2.5 hours for Dogs B and E respectively, and a second, faster component with half times of 0.68 and 0.75 hours respectively. The faster half times are in close agree-

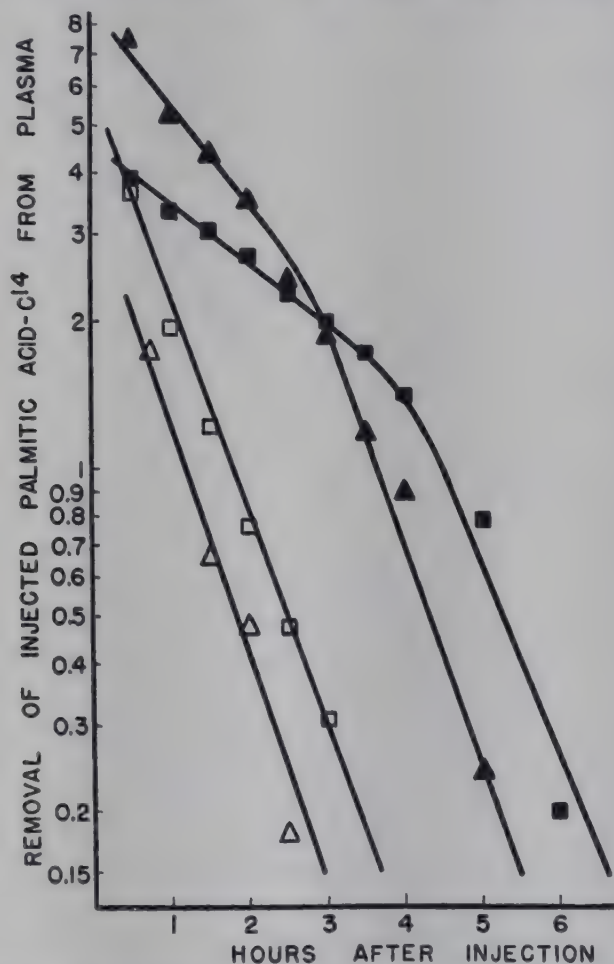


FIG. 2. Removal of injected palmitic acid- C^{14} from the plasma of normal and hepatectomized dogs following injection of tripalmitin containing palmitic acid- C^{14} . (Semilogarithmic plot.) For an explanation, see the text. □ normal Dog C, △ normal Dog F, ▲ hepatectomized Dog B, and ■ hepatectomized Dog E.

ment with those found for the normal dogs. The slower component could mean that the extrahepatic tissues are limited in their capacity to handle fatty acids and that the amount of fatty acids present had saturated the removal mechanism. That the later, faster rate of removal in the hepatectomized dogs was practically identical with the rate in the normal dogs raises the interesting question whether the mechanisms concerned with the removal of the labeled fatty acid from the blood stream are the same in liver and in extrahepatic tissues.

C¹⁴O₂ in Expired Air after Injection of Palmitic Acid-C¹⁴ in Normal and Hepatectomized Dogs

Fig. 3 shows the cumulative output of C¹⁴O₂ by the two normal (Dogs C and F) and the two hepatectomized dogs (Dogs B and E). At the end of the 7 hours, the total CO₂ eliminated by the normal dogs contained 11.3

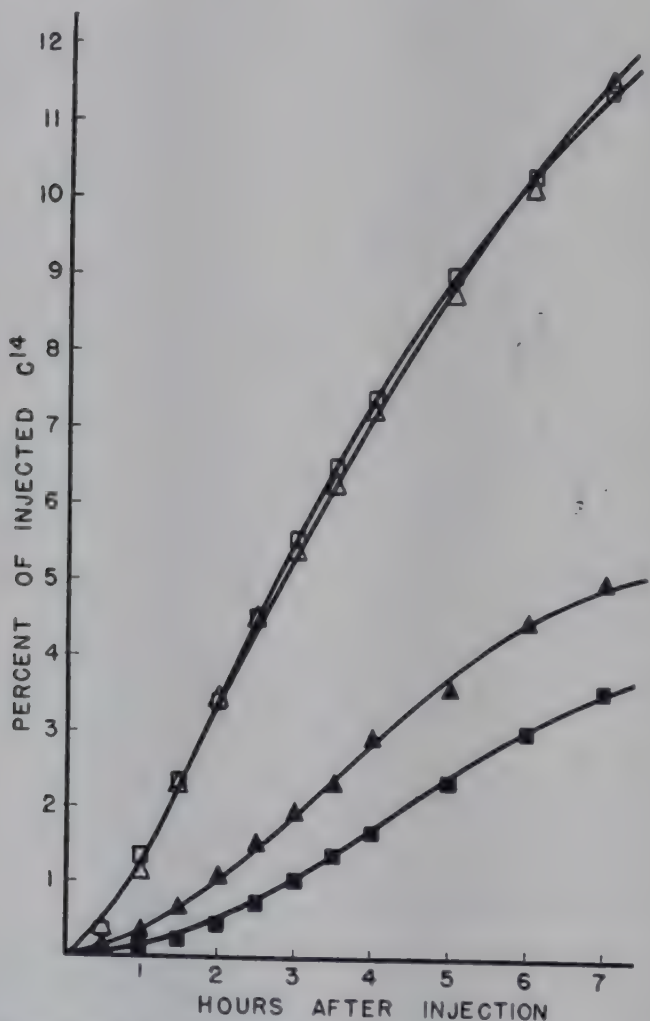


FIG. 3. C¹⁴O₂ exhaled by normal and hepatectomized dogs following intravenous injection of tripalmitin containing palmitic acid-1-C¹⁴. □ normal Dog C, △ normal Dog F, ▲ hepatectomized Dog B, and ■ hepatectomized Dog E.

and 11.5 per cent, respectively, of the injected C¹⁴; in this time, 3.6 and 5.0 per cent, respectively, were excreted by the hepatectomized dogs.

DISCUSSION

In the present study, the extent of oxidation of a sample of C¹⁴-labeled palmitic acid was compared in the normal dog and in the dog intact except

for the absence of a single organ, the liver. Each normal and each hepatectomized dog received, intravenously, a total of 1.5 gm. of fat containing 600 mg. of labeled tripalmitin. In the absence of the liver, the oxidation of the injected palmitic acid was reduced by 60 per cent. But it cannot be inferred that 40 per cent of the total $C^{14}O_2$ exhaled by the *normal* dogs resulted from the direct oxidation of the injected palmitic acid- C^{14} by their extrahepatic tissues. The removal of the palmitic acid from the circulation was more rapid in the normal than in the liverless dog. Consequently, when the liver is excised, greater amounts of the injected fatty acids become available to the extrahepatic tissues. Hence, although we cannot evaluate the amount of the injected palmitic acid- C^{14} that was directly converted to CO_2 by the extrahepatic tissues of the normal dogs, we do know that it was less than 40 per cent. Indeed, the observations recorded here do not rule out the possibility that all of the injected palmitic acid- C^{14} was oxidized in the liver of the normal dog.

It must be recognized that the experiments carried out here are unphysiological in the sense that the injected palmitic acid was not in the form in which it exists in the animal body. These experiments do prove, however, that extrahepatic tissues can oxidize palmitic acid directly. Since it has recently been shown, with the aid of C^{14} -glucose,¹ that muscle slices synthesize higher fatty acids, it seems likely that the oxidation of higher fatty acids is a normally occurring process in muscle.

The question as to the availability of *plasma* fatty acids for direct oxidation by extrahepatic tissues remains to be considered. With the aid of P^{32} as a labeling agent, it was shown that in the normal dog plasma phospholipides are completely turned over in 6 to 10 hours.² When the liver was excluded from the circulation, the time required for the complete turnover of plasma phospholipides was prolonged to as much as 160 hours (11). This indicates that plasma phospholipide fatty acids are not available for oxidation by tissues other than the liver. The availability of plasma non-phospholipide fatty acids for direct oxidation by extrahepatic tissues is at present under investigation.

SUMMARY

1. Palmitic acid-1- C^{14} was injected intravenously into normal and liverless dogs and its conversion to CO_2 was measured. Approximately 11 per cent of the C^{14} was expired as CO_2 in 7 hours by the normal dog, and about 4 per cent in the same length of time by the liverless dog.

¹ Chernick, S., and Masoro, E. J., unpublished observations.

² Using doubly labeled phospholipides (P^{32} and C^{11}), Weinman has shown that the turnover times for phosphate and fatty acid moieties are similar (unpublished observations).

2. Evidence is presented for the view that less than 40 per cent of the C¹⁴O₂ exhaled by the normal dog could have resulted from direct oxidation of the labeled palmitic acid by all of its extrahepatic tissues.

3. The extent of direct oxidation of higher fatty acids by extrahepatic tissues is discussed.

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SITE OF FORMATION OF PLASMA PHOSPHOLIPIDES STUDIED WITH C¹⁴-LABELED PALMITIC ACID*

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In earlier reports it was shown, with the aid of radioactive phosphorus, that surviving slices of liver (1), kidney (2), brain (3), and muscle¹ can incorporate inorganic phosphate into phospholipides, and it would therefore appear that phospholipide formation is not restricted to any one tissue. Despite the widespread occurrence of this reaction, Fishler *et al.* (4) observed that the incorporation of injected, labeled phosphate into plasma phospholipides does not occur in the absence of the liver. Thus 6 hours after the injection of P³² into both normal and liverless dogs the percentages of the labeled phosphate recovered as plasma phospholipide were 40 to 70 times as great in the normal as in the hepatectomized dogs.

The finding that isolated surviving liver slices can incorporate labeled phosphate into phospholipide molecules can leave no doubt that liver tissue possesses a mechanism for the synthesis of the phosphate-alcohol ester bond and, presumably, this is the reaction performed by the liver when injected P³² is incorporated into *plasma* phospholipides. The question as to whether the liver is also a site for the incorporation of *fatty acids* into plasma phospholipide was left unanswered.

This phase of plasma phospholipide formation has been studied here with C¹⁴-labeled palmitic acid. In addition, by the use of this labeled fatty acid, we have obtained new and significant evidence on the fatty acid transport function of phospholipides.

EXPERIMENTAL

The results shown in Tables I and II, and in Fig. 1, were obtained from the normal and hepatectomized dogs described in the preceding paper (5). The operative procedures have been described there.

Methods of Analyses

Extraction of Lipides; Plasma—Whole blood was withdrawn from the femoral artery into a heparinized syringe. A known volume of plasma

* Aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

¹ Fishler, M. C., unpublished observations.

(usually 3 to 5 cc.) was transferred into 30 volumes of 95 per cent ethyl alcohol and thoroughly mixed. Enough freshly distilled ethyl ether was added to the alcoholic suspensions of plasma to make the solvent consist of 3 parts alcohol and 1 part ether. The mixture was extracted on a water bath at 55–60° for 1 hour. The alcohol-ether extract was then poured through a filter paper into a concentration flask. The solid resi-

TABLE I

Recovery of Intravenously Injected Palmitic Acid-C¹⁴ in Lipide Fractions of Plasma and Tissues of Normal Dog C

600 mg. of tripalmitin containing palmitic acid-1-C¹⁴ were injected and the dog sacrificed 7 hours later. The organs, with the exception of heart and lungs, were perfused with physiological saline before the samples were taken. The dog weighed 8.7 kilos.

Tissue	Organ weight	Lipide content of tissue		Per cent of injected C ¹⁴ recovered as				Specific activity of phospholipide fatty acids*
		Phospholipide fatty acids	Non-Phospholipide fatty acids	Phospholipide fatty acids		Non-Phospholipide fatty acids		
				Per whole organ	Per 100 gm. tissue	Per whole organ	Per 100 gm. tissue	
	gm.	mg. per 100 gm. wet wt.	mg. per 100 gm. wet wt.					
Liver.....	325	1380	660	17.8	5.48	14.4	4.43	3.97 × 10 ⁻³
Lung.....	63.3	1360	1330	1.56	2.46	1.44	2.28	1.82 × 10 ⁻³
Heart.....	42.0	943	3600	0.28	0.67	0.21	0.50	0.71 × 10 ⁻³
Kidney.....	51.0	1120	1280	0.50	0.98	0.12	0.24	0.88 × 10 ⁻³
Muscle†.....		462	1840		0.19		0.31	0.41 × 10 ⁻³
Small intestine.....	310	860	970	3.46	1.12	0.95	0.31	1.29 × 10 ⁻³
Mucosa of small intestine†...		1020	565		1.52		0.47	1.50 × 10 ⁻³
Plasma.....		240	400		0.43		0.75	1.60 × 10 ⁻³

* The specific activity is expressed here as per cent of the injected C¹⁴ recovered per mg. of phospholipide fatty acids.

† Gastrocnemius.

‡ Mucosa was scraped from a piece of intact small intestine. A separate intact sample of small intestine was used for small intestine lipide analyses.

due was transferred to the filter paper by ethyl ether washes, when it was again washed thoroughly with ethyl ether. All ether washes were combined with the original alcohol-ether extract. The solid residue was discarded.

Tissues—Immediately after the dog was sacrificed, the abdomen was opened and the muscles and abdominal organs were perfused with physiological saline. Heart and lung were washed free of adhering blood.

Whole organs were excised and weighed. Individual samples of the organs were minced in 95 per cent ethyl alcohol for about 30 seconds in the Waring blender. The minced samples were stored in a refrigerator until analyzed.

Enough ethyl ether was added to the tissue minces (in alcohol) to make the solvent a 3:1 alcohol-ether mixture. The minces were extracted for 2 hours in a water bath at 55-60°. The alcohol-ether extract was

TABLE II

Recovery of Intravenously Injected Palmitic Acid-C¹⁴ in Lipide Fractions of Plasma and Tissues of Hepatectomized Dog E

600 mg. of tripalmitin containing palmitic acid-1-C¹⁴ were injected as soon as hepatectomy was completed. The animals were sacrificed 7 hours later. The organs, with the exception of heart and lungs, were perfused with physiological saline before the samples were taken. The dog weighed 7.0 kilos.

Tissue	Organ weight	Lipide content of tissue		Per cent of injected C ¹⁴ recovered as				Specific activity of phospholipide fatty acids*
		Phospho- pho- lipide fatty acids	Non-Phos- pho- lipide fatty acids	Phospho- lipide fatty acids		Non-Phos- pholipide fatty acids		
				Per whole organ	Per 100 gm. tissue	Per whole organ	Per 100 gm. tissue	
	gm.	mg. per 100 gm. wet wt.	mg. per 100 gm. wet wt.					
Lung.....	57.3	1610	890	1.62	2.82	2.04	3.56	1.76 × 10 ⁻³
Heart.....	48.3	1390	1760	0.77	1.59	1.45	3.00	1.15 × 10 ⁻³
Kidney.....	40.8	1510	1230	0.50	1.22	0.15	0.37	0.82 × 10 ⁻³
Muscle†.....		892	2420		0.11		0.29	0.12 × 10 ⁻³
Small intestine.....	228	870	438	1.31	0.57	0.38	0.17	0.66 × 10 ⁻³
Mucosa of small intestine†..		1060	630		0.66		0.23	0.62 × 10 ⁻³
Plasma.....		130	320		0.03		7.36	0.34 × 10 ⁻³

* The specific activity is expressed here as per cent of the injected C¹⁴ recovered per mg. of phospholipide fatty acids.

† Gastrocnemius.

‡ Mucosa was scraped from a piece of intact small intestine. A separate intact sample of small intestine was used for small intestine lipide analyses.

decanted through a filter paper into a concentration flask. A fresh sample of a 3:1 alcohol-ether mixture was added to the tissue residue, and the residue was reextracted for 2 hours at 55-60°. This second alcohol-ether extract was poured through the same filter paper used for the first decantation. The solid tissue residue was then transferred to the filter paper by small ethyl ether washes, and washed thoroughly with ethyl ether. These washings were collected in the concentration flask and com-

bined with the alcohol-ether extracts obtained previously. The solid residue was then extracted overnight, in a Soxhlet flask, with ethyl ether. This ethyl ether extract was combined with the previous extracts in the concentration flask.

Separation of Lipides into Phospholipide and Non-Phospholipide Fractions—The extracts were concentrated to a low volume (2 to 3 cc.) under

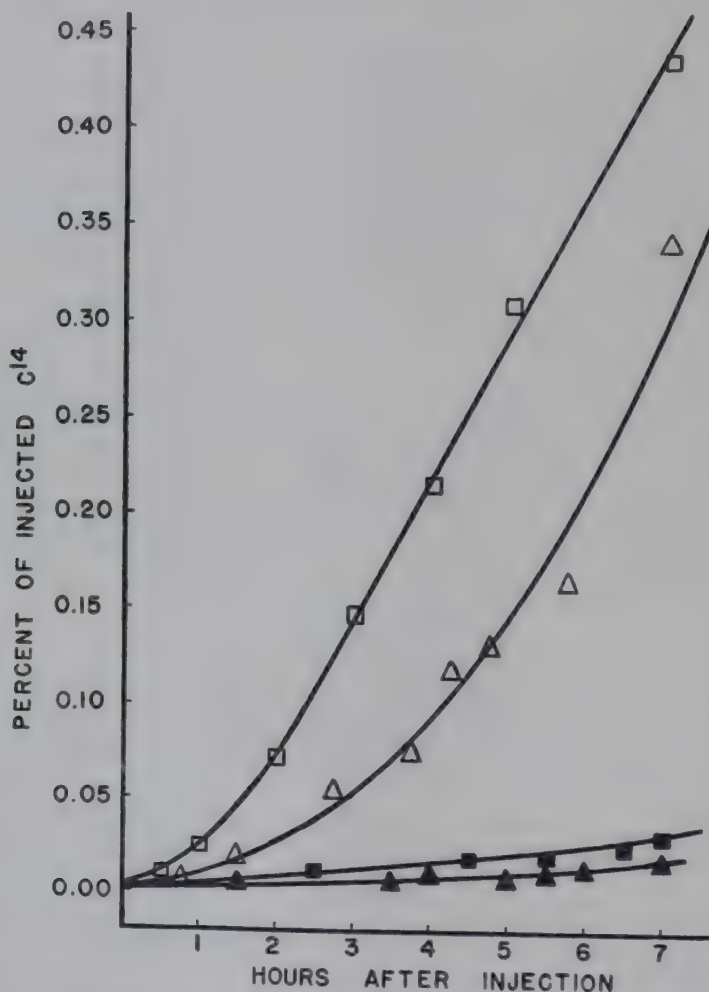


FIG. 1. The incorporation of injected palmitic acid-C¹⁴ into plasma phospholipides. The ordinate represents per cent recovery of injected C¹⁴ in the phospholipide fatty acids per 100 cc. of plasma. □ normal Dog C, △ normal Dog F, ▲ hepatectomized Dog B, and ■ hepatectomized Dog E.

a vacuum. The last 75 to 100 cc. were removed under a CO₂ atmosphere. The concentrate was transferred to an Erlenmeyer flask equipped with a side arm (1), and the lipides were thoroughly extracted with petroleum ether. The petroleum ether extracts were evaporated to a low volume (about 0.5 to 1 cc.), and the phospholipides were precipitated by adding 30 cc. of anhydrous acetone and 10 to 30 drops (depending on the amount

of phospholipides present) of a saturated solution of MgCl_2 in absolute alcohol. The mixture was centrifuged and the acetone supernatant (non-phospholipide fraction) was decanted. The precipitated phospholipides were thoroughly washed twice with fresh anhydrous acetone and, after centrifugation, the acetone washes were combined with the acetone supernatant referred to above.

The non-phospholipide fraction was transferred to an Erlenmeyer flask equipped with a side arm and evaporated almost to dryness. 3 to 4 cc. of 95 per cent ethyl alcohol were then added. The non-phospholipide fraction was saponified by adding 1 cc. of 90 per cent aqueous KOH for each 500 mg. of fat and heating on a steam bath for 30 minutes. The mixture was next acidified with 5 N H_2SO_4 , and the fatty acids were extracted with ethyl ether.

3 to 4 cc. of 95 per cent ethyl alcohol were added to the MgCl_2 -phospholipide precipitate, obtained as described above. The phospholipides were saponified by adding 1 ml.² of 90 per cent aqueous KOH for each 500 mg. of phospholipide and heating the mixture for 1 hour on a steam bath. The mixture was acidified with 5 N H_2SO_4 , and the free fatty acids were extracted with ethyl ether. The remaining acidified aqueous phase was then freed of dissolved ethyl ether by heating, and set aside for phosphorus determination. Fatty acids were determined by a modification of the oxidative procedure of Okey (6). Phosphorus was determined by King's method (7).

Determination of C^{14} Content of Fatty Acids—The C^{14} contents of the phospholipide and non-phospholipide fatty acid fractions were determined by the direct mount technique described by Entenman *et al.* (8).

Results

Recovery of Injected Palmitic Acid- C^{14} in Fatty Acid Fraction of Plasma Phospholipides

Fig. 1 shows the incorporation of the palmitic acid- C^{14} into phospholipide fatty acids of plasma during the 7 hours after the injection. The values are expressed as the percentages of the injected palmitic acid- C^{14} recovered in phospholipide fatty acids per 100 cc. of plasma. The difference between the normal and liverless dogs is quite striking. If it is assumed that the dogs used in these experiments had plasma volumes of 500 cc., then about 2 per cent of the injected C^{14} was recovered as plasma phospholipides in the normal dogs and about 0.1 per cent in the liverless dogs.

² The minimal quantity of 90 per cent KOH used was 1 cc.

Recovery of Injected Palmitic Acid-C¹⁴ in Fatty Acid Fraction of Tissues

7 hours after the injection of the emulsion containing palmitic acid-C¹⁴, the dogs were sacrificed by a cardiac injection of nembutal. Various tissues were excised and analyzed for their content of phospholipide and non-phospholipide fatty acid-C¹⁴. The results for one of the normal and one of the hepatectomized dogs are recorded in Tables I and II. The results are expressed as (a) percentages of the injected C¹⁴ recovered as phospholipide fatty acids and non-phospholipide fatty acids per whole organ or per 100 gm. of tissue, and (b) specific activities of phospholipide fatty acids.

In view of the failure of the hepatectomized dog to incorporate the injected palmitic acid-C¹⁴ into plasma phospholipide, it is of interest to note that this preparation did incorporate the C¹⁴ into phospholipide fatty acids of the small intestine and its mucosa, the lung, heart, kidney, and skeletal muscle. The phospholipides of those five tissues contained approximately 10 per cent of the injected palmitic acid-C¹⁴. To obtain this value, it was assumed that skeletal muscle constitutes 40 per cent of the body weight.

DISCUSSION

When inorganic P³² is injected into the hepatectomized dog, the amounts of the isotope recovered in plasma phospholipides are negligible, as compared with recoveries in normal dogs (4). When palmitic acid-1-C¹⁴ is injected into dogs deprived of their liver, the recovery of isotopic fatty acids in plasma phospholipide is again negligible in comparison with what occurs in the normal dog. Thus, by the use of two labeled moieties of the phospholipide molecule, we have demonstrated that the liver is the principal site for the synthesis of plasma phospholipides.

In the liverless dog, about 10 per cent of the C¹⁴ was recovered as phospholipide fatty acids in the various tissues studied. This observation reinforces our earlier conclusion that appreciable amounts of the phospholipides formed in extrahepatic tissues are not delivered to plasma (4).

The above considerations again bring to the fore the question of the rôle of phospholipides in fatty acid metabolism. The fact that the injected, labeled palmitic acid-C¹⁴ was incorporated into phospholipide fatty acids of the muscle, the kidney, and the small intestine, but not into plasma phospholipides, can leave no further doubt that, in the dog, phospholipides of the extrahepatic tissues are not normally vehicles for the transport of fatty acids from one tissue to another.

It was pointed out elsewhere (9) that the excision of the liver from the circulation abruptly halts, or brings about a pronounced reduction in,

the plasma phospholipide utilization. Thus, both synthesis and utilization of plasma phospholipides occur principally, if not solely, in the liver. Such localization is also true for each of the extrahepatic tissues examined here. It would therefore appear that the metabolic cycle of each phospholipide molecule, from synthesis to disruption, occurs in a single tissue.

SUMMARY

1. Palmitic acid-1-C¹⁴ was injected intravenously into both normal and hepatectomized dogs, and the recovery of labeled fatty acid in phospholipides of plasma and tissues was determined.

2. 7 hours after the injection, about 2 per cent of the C¹⁴ was found as phospholipide fatty acids of plasma in the normal dogs. About 0.1 per cent was so recovered in the liverless dogs. The liver is, therefore, the principal site for the formation of fatty acid ester bonds of plasma phospholipide molecules.

3. In the liverless dogs, isotopic fatty acids were recovered in the phospholipides of heart, skeletal muscle, kidney, small intestine and its mucosa, and lung. The failure of these tissues to deliver appreciable amounts of isotopic phospholipide fatty acids to the plasma of the liverless dog demonstrates that, in this animal, the phospholipides synthesized by the tissues above are not normally concerned with the transport of fatty acids from one organ to another.

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RELATIVE RATES OF CONVERSION OF THE VARIOUS CARBON ATOMS OF PALMITIC ACID TO CARBON DIOXIDE BY THE INTACT RAT*

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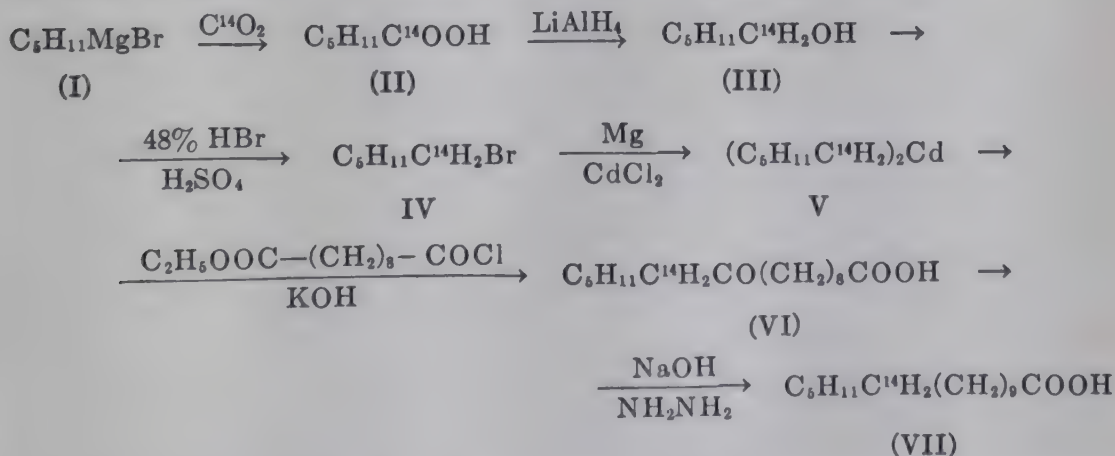
In a recent report from this laboratory it was shown that intravenously injected tripalmitin containing palmitic acid labeled with C^{14} at its 6th carbon was rapidly broken down, about 50 per cent of the C^{14} being expired as CO_2 in 24 hours (1). By employing this route of administration, which circumvents the difficulties imposed by absorption from the intestinal tract, it became feasible for the first time to compare, *in the intact animal*, the rates of oxidation of several of the carbon atoms of a physiologically occurring fatty acid. In the present investigation, palmitic acid that had been labeled with C^{14} either at the carboxyl, at the 6th, or at the 11th carbon atom was administered to rats intravenously as tripalmitin, and the elimination of the C^{14} in the expired CO_2 was studied.

EXPERIMENTAL

Synthesis of Labeled Palmitic Acids

Palmitic Acid-1- C^{14} and Palmitic Acid-6- C^{14} —The synthesis of these two palmitic acids has been described in an earlier report (2).

Palmitic Acid-11- C^{14} —The synthesis of palmitic acid containing C^{14} in its 11th carbon atom involved the following steps:



* Aided by a grant from the American Cancer Society, as recommended by the Committee on Growth of the National Research Council.

Caproic Acid-1-C¹⁴ (II)—The Grignard reagent was prepared in a nitrogen atmosphere from 4.25 gm. (0.175 mole) of magnesium turnings and 25.52 gm. (0.168 mole) of amyl bromide in 100 cc. of anhydrous ether. An aliquot of this solution was titrated and the concentration was found to be 0.0152 mole of Grignard reagent per cc.

A volume of 62 cc. (0.0921 mole) of Grignard solution was carbonated with radioactive carbon dioxide generated from 16.5 gm. (0.0835 mole) of barium carbonate containing a total activity of approximately 5 mc. by the procedure of Dauben, Reid, and Yankwich (3). The acid was isolated in the usual way and distilled. A yield of 8.31 gm. (85.5 per cent), b.p. 200–202°, n_D^{20} 1.4143, was obtained.

Hexanol-1-C¹⁴ (III)—Carboxyl-labeled caproic acid (8.31 gm., 0.0716 mole) was reduced to hexanol by dissolving the acid in 100 cc. of anhydrous ether and adding the solution dropwise to a suspension of 5.45 gm. (0.1432 mole) of lithium aluminum hydride in 120 cc. of anhydrous ether so that the ether was refluxed gently (4). Stirring was continued for 15 minutes at room temperature after complete addition. The reaction mixture was decomposed by adding ice water and then 10 per cent sulfuric acid. The aqueous phase was saturated with sodium chloride and extracted with ether. Sodium chloride was added to reduce the solubility of hexanol in water. The ether extracts were washed with 4 per cent aqueous sodium hydroxide solution, and dried over anhydrous sodium sulfate. The alcohol was distilled, b.p. 155–156°, $n_D^{24.3}$ 1.4161, yielding 6.53 gm. (89.5 per cent based on caproic acid; 76.7 per cent based on BaCO₃).

n-Hexyl Bromide-1-C¹⁴ (IV)—Hexanol was brominated, according to the procedure described for octyl bromide (5), with 6.53 (0.064 mole) of *n*-hexanol, 28 gm. of 48 per cent hydrobromic acid, and 7.3 gm. of concentrated sulfuric acid. The reaction mixture was refluxed for 6 hours, with the bath temperature maintained between 110–120°, cooled to room temperature, and diluted with water. The bromide layer was separated and washed with concentrated sulfuric acid and water, and dried over anhydrous potassium carbonate. All the aqueous washes were extracted with ether. The ether extracts were then dried over potassium carbonate and combined with the bromide. The solvent was removed and the bromide was distilled, giving a yield of 8.60 gm. (81.3 per cent based on hexanol; 62.3 per cent over-all from barium carbonate), b.p. 154–156°, n_D^{18} 1.4492.

Hexadecanoic Acid-11-C¹⁴ (VII)—The Grignard reagent was prepared from 8.60 gm. (0.0521 mole) of *n*-hexyl bromide in 100 cc. of dry ether and 1.32 gm. (0.0543 mole) of magnesium turnings. Cadmium chloride (5.24 gm., 0.0286 mole) was added to the Grignard mixture and stirred under a reflux until a negative Gilman test was obtained. The ether was distilled and replaced with 100 cc. of dry benzene. A benzene solution

of 9-carboxynonanoyl chloride (16.85 gm. titrated for 77 per cent acid chloride, 0.0527 mole) was added with stirring to the suspension of dialkyl cadmium compound and the mixture heated under a reflux for an hour (6). The reaction mixture was decomposed as usual, and the keto acid was isolated by saponification of the ester with 5 gm. of potassium hydroxide in 125 cc. of methanol for 1 hour. The diethyl sebacate believed to be present in the half ester acid chloride would also hydrolyze and be extracted with the keto acid. The alkaline mixture was extracted with ether to remove neutral compounds and acidified. The acids were extracted with ether. The ether extracts were dried over Drierite, and the solvent was distilled. A residue of 11 gm. remained (keto acid plus sebacic acid).

The crude keto acid was reduced by the modified Wolff-Kishner method (7) by heating with 3.50 cc. of 100 per cent hydrazine hydrate, 72.3 cc. of diethylene glycol, and 1.04 gm. of sodium hydroxide for 1 hour at 150° and for 4 hours at 220°. The reaction mixture was acidified with dilute hydrochloric acid while still warm. It was then cooled and extracted with ether. The ether extracts were washed with water and saturated aqueous sodium chloride, and treated with norit. The ether was evaporated, and the residue was crystallized from acetone-water. The solid material was collected and dissolved in hot *n*-hexane. The insoluble material was filtered, and the mother liquor was cooled in an ice bath. White, shiny crystals were collected weighing 2.49 gm. (10.5 per cent over-all based on BaCO₃, 18.6 per cent based on bromide), m.p. 61–62°.

9-Carboethoxynonanoyl Chloride—The acid chloride was prepared by treating ethyl hydrogen sebacate with excess thionyl chloride at 30–40° for 3 hours. Excess thionyl chloride was removed under reduced pressure, and the product was distilled, b.p. 145–147° (2 to 3 mm.).

Preparation of Tripalmitin Emulsions

Esterification—The fatty acids were esterified with glycerol by a modification of the method of Feuge *et al.* (8).

Emulsification—All emulsions contained 2 per cent tripalmitin, 2 per cent olive oil, 1 per cent glycerol monostearate, 5 per cent glucose, and 90 per cent distilled water, and were prepared by a modification of the method described by Goldman *et al.* (9). Weighed quantities of the tripalmitin, olive oil, and glycerol monostearate were heated in a small test-tube until liquefaction occurred, and the required quantity of a 5 per cent solution of glucose was then added. The test-tube was immersed in water kept between 70–75°, and the mixture was agitated violently for 10 minutes by means of a propeller-shaped stirrer attached to a high speed motor. Particle size of each emulsion was determined by microscopic examina-

tion. The average particles of the emulsions used in Experiment I were about $3\ \mu$ in diameter. The stirrer was redesigned for Experiment II to give greater shearing action, and the particles of the resulting emulsion averaged about $1\ \mu$. All emulsions were injected within an hour after their preparation.

Treatment of Animals and Collection of $C^{14}O_2$ —Female rats of the Long-Evans strain, weighing from 160 to 200 gm., were used in both experiments. Rats that had been fasted for 24 hours were lightly anesthetized with ether and then injected with 1.0 cc. of an emulsion. The emulsion was administered slowly via the foot vein, the total time for the injection being about 1 minute. Each rat was placed in a narrow, elongated glass

TABLE I
Expiration of Carboxyl, 6th, and 11th Carbons of Intravenously Administered Palmitic Acid As CO_2 ; Experiment I

Rat No.	Weight	Carbon of palmitic acid labeled	Counts per min. injected	Per cent administered C^{14} recovered as CO_2 during							
				0-1 hr.	1-1½ hrs.	1½-2½ hrs.	2½-3½ hrs.	3½-4 hrs.	4-12 hrs.	12-24 hrs.	0-24 hrs.
	gm.										
1	172	Carboxyl	1120×10^3	0.9	3.2	2.8	2.8	2.5	14.5	13.6	40.3
2	162	"	1120×10^3	0.8	2.4	2.3	2.0	2.0	10.1*		
3	176	"	1120×10^3	1.2	2.5	3.6	2.7	2.2	17.1	15.3	44.6
4	178	6th	1160×10^3	1.0	2.5	2.7	2.6	2.2	17.2	14.6	42.8
5	162	6th	1160×10^3	2.1	3.8	3.2	2.9	2.9	14.3	13.7	42.9
6	175	6th	1160×10^3	3.4	4.2	3.8	4.2	2.4	17.3	12.8	48.1
7	164	11th	549×10^3	0.6	1.7	2.0	3.7	3.1	18.6	16.1	45.8
8	180	11th	625×10^3	0.7	2.2	2.0	1.7	2.0	13.2		

* The collection period in this case was 4 to 10 hours.

cage which was ventilated by CO_2 -free air, and the expired CO_2 was collected in towers containing sodium hydroxide solutions as described by Lerner *et al.* (1). During the experiment, the rats had access to water but food was withheld. Urine was collected only in Experiment II.

Aliquots of the Na_2CO_3 - $NaOH$ solutions were used for the determination of total CO_2 and $C^{14}O_2$ expired. The methods employed were those described by Entenman *et al.* (10).

Results

Experiment I—Eight rats were used in this experiment. Three were injected with an emulsion containing tripalmitin-1- C^{14} , three with tripalmitin-6- C^{14} , and two with tripalmitin-11- C^{14} . CO_2 was collected for

TABLE II
Expiration of Carboxyl, 6th, and 11th Carbons of Intravenously Administered Palmitic Acid As CO_2 ; Experiment II

Rat No.	Weight	Carbon of palmitic acid labeled	Counts per min. injected	Per cent administered C ¹⁴ recovered as CO ₂ during																
				0- $\frac{1}{2}$ hr.	$\frac{1}{2}$ -1 hr.	1-1 $\frac{1}{2}$ hrs.	1 $\frac{1}{2}$ -2 hrs.	2-2 $\frac{1}{2}$ hrs.	2 $\frac{1}{2}$ -3 hrs.	3 $\frac{1}{2}$ -4 hrs.	4-4 $\frac{1}{2}$ hrs.	4 $\frac{1}{2}$ -5 hrs.	5-6 hrs.	6-8 hrs.	8-12 hrs.	12-23 hrs.	23-24 hrs.	0-24 hrs.		
	gm.																			
9	196	Carboxyl	1790 $\times$ 10 ³	4.0	9.1	3.9	6.6	3.4	3.3	4.0	3.9	3.6	2.3	1.7	3.7	4.9	5.6	8.8	0.6	69.4
10	192	"	1790 $\times$ 10 ³	3.4	7.8	3.7	4.3	4.5	2.7	3.3	3.3	4.1	2.1	2.0	3.6	5.3	6.9	10.3	0.6	67.9
11	178	"	1790 $\times$ 10 ³	2.4	5.6	4.5	4.2	3.3	3.3	3.9	3.8	3.5	2.6	1.7	3.2	4.9	6.9	8.8	0.5	63.1
				0- $\frac{1}{2}$ hr.	$\frac{1}{2}$ -1 hr.	1-1 $\frac{1}{2}$ hrs.	1 $\frac{1}{2}$ -2 hrs.	2-2 $\frac{1}{2}$ hrs.	2 $\frac{1}{2}$ -3 hrs.	3 $\frac{1}{2}$ -4 hrs.	4-4 $\frac{1}{2}$ hrs.	4 $\frac{1}{2}$ -5 hrs.	5-6 hrs.	6-8 hrs.	8-12 hrs.	12-23 hrs.	23-24 hrs.	0-24 hrs.		
12	164	6th	737 $\times$ 10 ³	2.2	5.0	3.6	2.8	2.6	2.2	1.9	2.9	2.4	3.3	6.7	6.3	6.7	14.0	2.3	0.4	65.3
13	199	6th	737 $\times$ 10 ³	0.9	3.8	3.4	3.2	2.5	2.0	1.9	2.9	2.3	3.2	6.4	6.6	5.5	12.3	2.1	0.4	59.4
14	180	6th	737 $\times$ 10 ³	1.3	4.1	2.6	3.3	3.0	2.7	3.4	3.8	3.3	4.8	6.2	5.8	5.9	11.9	1.7	0.6	64.4
				0- $\frac{1}{2}$ hr.	$\frac{1}{2}$ -1 hr.	1-1 $\frac{1}{2}$ hrs.	1 $\frac{1}{2}$ -2 hrs.	2-2 $\frac{1}{2}$ hrs.	2 $\frac{1}{2}$ -3 hrs.	3 $\frac{1}{2}$ -4 hrs.	4-4 $\frac{1}{2}$ hrs.	4 $\frac{1}{2}$ -5 hrs.	5 $\frac{1}{2}$ -7 $\frac{1}{2}$ hrs.	7 $\frac{1}{2}$ -9 $\frac{1}{2}$ hrs.	9 $\frac{1}{2}$ -12 hrs.	12-23 hrs.	23-24 hrs.	0-24 hrs.		
15	163	11th	826 $\times$ 10 ³	4.7	7.5	3.5	2.9	2.6	2.1	2.2	2.7	2.6	2.2	5.4	5.8	4.7	4.6	12.1	0.9	66.5
16	176	11th	826 $\times$ 10 ³	2.4	7.4	5.3	4.5	3.8	3.0	2.2	2.5	3.0	2.7	5.9	5.7	4.1	5.4	10.6	0.7	69.2
17	190	11th	826 $\times$ 10 ³	2.5	5.2	3.8	4.3	3.2	3.0	2.1	2.2	2.5	2.2	5.6	5.7	5.0	5.0	10.6	0.6	63.5

24 hours, and during that period seven separate samples were obtained. The shortest collection interval was 45 minutes.

The percentages of the injected C^{14} recovered in the expired CO_2 at various time intervals are recorded in Table I. The specific activity-time relations of the expired CO_2 are shown in Fig. 1.

Experiment II (Table II and Fig. 2)—Nine rats were used in this experiment. Groups of three were injected with tripalmitin emulsions in which the palmitic acid was again labeled at either the carboxyl, the 6th, or the 11th carbon. In order to determine more exactly the time of oc-

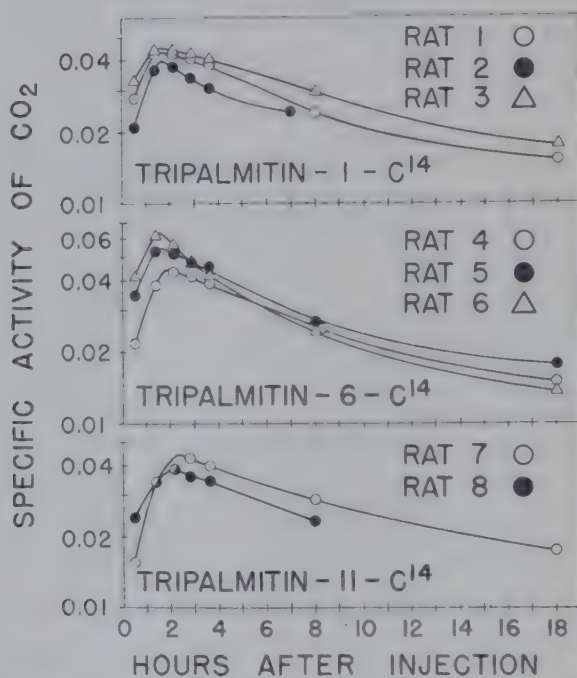


FIG. 1. Semilogarithmic plot of the specific activities of expired CO_2 against time. Specific activity refers to the percentage of the injected palmitic acid- C^{14} recovered in expired air per mg. of CO_2 -carbon. The data are obtained from Experiment I.

currence of the maximum specific activity and the rates of $C^{14}O_2$ elimination, samples were collected at more frequent intervals than in Experiment I. A total of sixteen samples was collected in the first 24 hours after the injections; some of the intervals were as short as 20 minutes.

Total $C^{14}O_2$ Elimination for 24 Hours—In Experiment I, from 40 to 48 per cent of the injected C^{14} was recovered in 24 hours. The position of the label did not seem to influence the $C^{14}O_2$ recovered.

In Experiment II, the 24 hour (cumulative) values ranged from 59 to 69 per cent. The values found for the rats injected with the differently labeled palmitic acids were in good agreement.

Since the particle size of the emulsion used in Experiment I (about $3\ \mu$ in diameter) differed from that used in Experiment II (about $1\ \mu$ in diameter), it would appear that the particle size has an influence on the rate of oxidation of the injected tripalmitin. For this reason the comparison

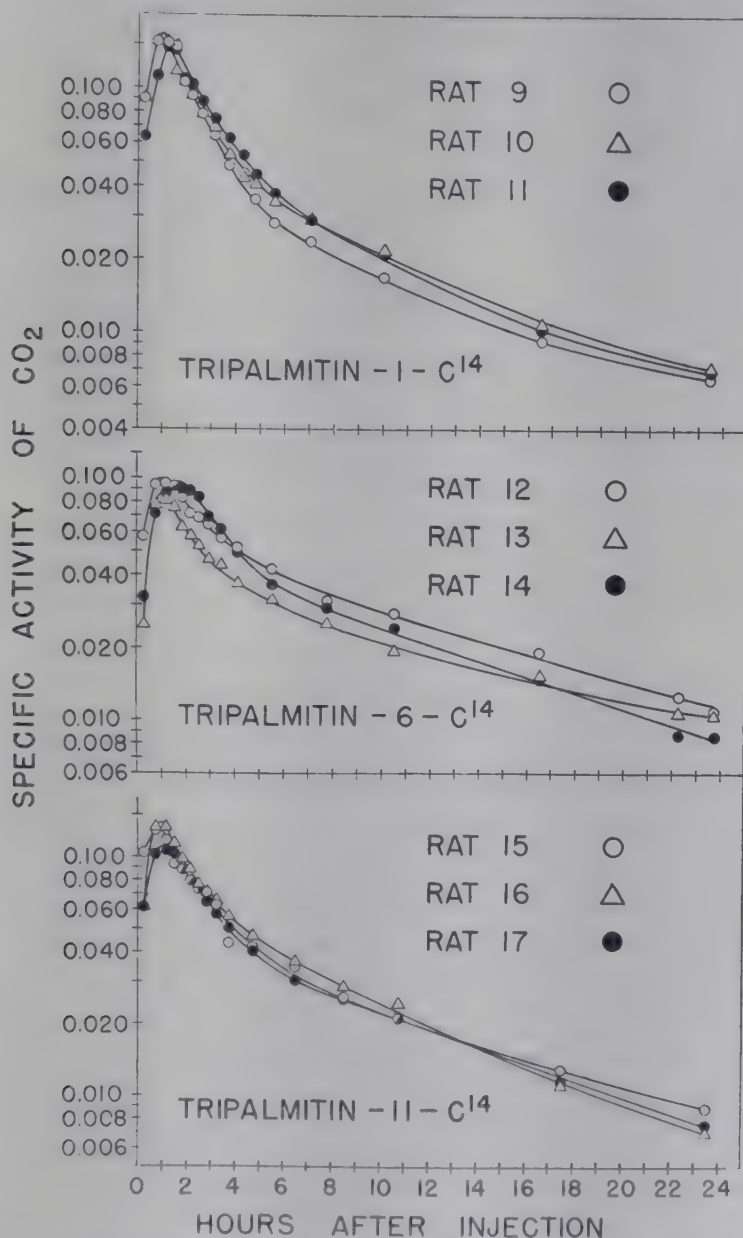


FIG. 2. Semilogarithmic plot of the specific activities of expired CO_2 against time. The data are obtained from Experiment II.

of the C^{14}O_2 eliminations resulting from the injection of the three differently labeled palmitic acids has been made separately for each experiment.

Specific Activity-Time Relations—As can be seen from Tables I and II, and from the specific activity-time curves (Figs. 1 and 2), the rates of

elimination of $C^{14}O_2$ were not uniform throughout the period of observation but rose rapidly after the injection until they reached a maximum, and then fell steadily. In Experiment I, the maximum specific activities were observed at 1.5 hours after injection of the C-6- and the carboxyl-labeled acids, and about 45 minutes later for the two rats which had received the C^{14} -11-labeled compound (Fig. 1). In Experiment II, maximum specific activities were observed about 1 hour after the injection, regardless of the position of the C^{14} in the palmitic acid molecule (Fig. 2).

It is of interest to note that the times of occurrence of the maximum specific activities remained fairly constant within each experiment, regardless of the location of the C^{14} .

The relative oxidative rates of the variously labeled palmitic acids were also compared by determining the actual rates of elimination of $C^{14}O_2$. Rate curves were constructed by determining the tangents of cumulative curves (cumulative $C^{14}O_2$ expired *versus* time) at various points and plotting these values against time. The rate curves so obtained did not appear to be influenced by the position of the C^{14} label.

Approximately 0.5 to 1 per cent of the injected C^{14} was recovered in the urine in 24 hours. This C^{14} was present primarily as urea. The urea was isolated by a modification of the method of Allen and Luck (11).

DISCUSSION

Regardless of whether the C^{14} was introduced into the carboxyl, the 6th, or the 11th carbon atom of the palmitic acid molecule, consistent differences in the percentages of the injected C^{14} recovered in the expired CO_2 were not detected. Furthermore, the times of occurrence of maximum specific activities of the expired CO_2 corresponded closely for the three differently labeled palmitic acids. These findings indicate that in the intact animal the carboxyl, the 6th, and the 11th carbons of palmitic acid are oxidized at rates that are not distinguishable from one another by the method used here. This would suggest that, once the process of breakdown is initiated, a palmitic acid molecule is disrupted in such a manner that all of its carbons are converted to CO_2 at about the same time.

In this discussion the oxidation of a fatty acid molecule will be considered in two phases, the first representing its breakdown to a 2-carbon fragment, as postulated by Weinhouse *et al.* (12), and the second, the subsequent utilization of the 2-carbon fragment either for synthetic purposes or for conversion to CO_2 via the tricarboxylic acid cycle.

According to this view oxidation of palmitic acid-6- C^{14} should give rise to acetate (or a similar 2-carbon fragment) in which the methyl group contains the label, while palmitic acid-1- C^{14} and palmitic acid-11- C^{14} each should form carboxyl-labeled acetate. When acetate is converted to CO_2

via the tricarboxylic acid cycle, fewer revolutions of the cycle are required for the conversion to CO_2 of the carboxyl than of the methyl carbons. Jones, however, has shown that injected acetate is so rapidly converted to CO_2 that measurable differences in the rates of C^{14}O_2 expiration after the administration of $\text{C}^{14}\text{H}_3\text{COOH}$ and $\text{CH}_3\text{C}^{14}\text{OOH}$ are not detectable.¹ Accordingly, once a palmitic acid molecule has been degraded to acetate, there should be no detectable difference in the subsequent rates at which the various carbon atoms are converted to CO_2 .

Stepwise β oxidation is the most generally accepted mode of fatty acid degradation, though the possibility of multiple alternate oxidation along the chain before its fragmentation has not been excluded. If breakdown of the fatty acid molecule occurs by stepwise fragmentation, one might expect to find a difference in the rates of elimination of the various carbons of the palmitic acid molecule. Our failure to find such differences in the intact rat might be construed to favor the view that the fatty acid molecule was split into fragments at various points simultaneously. But it must also be recognized that stepwise degradation may have occurred rapidly enough to make undetectable here the successive removal of 2-carbon fragments.

In view of the similarity in the rates of oxidation of the various carbon atoms of the palmitic acid molecule, one is led to the speculation that, once attached to its oxidative enzyme system, the palmitic acid molecule remains associated with it until the molecule is broken down to smaller units, possibly the 2-carbon fragments. Such a view has already been suggested by Weinhouse *et al.* (12) and by Barnes and Gurin (13).

The considerations presented above would appear to exclude the existence of a major portion of palmitic acid carbons as stable intermediates of shorter chain length than 16 in the direct path of oxidation of palmitic acid to CO_2 . They indicate rather that palmitic acid is oxidized predominantly to completion, or at least to a 2-carbon fragment, which is then either converted to CO_2 or utilized for synthetic reactions. Although there can no longer be any doubt that myristic and lauric acids can be derived from palmitic acid (14), our data indicate that, in the path of oxidation of palmitic acid, the number of molecules that become stabilized at lauric and myristic are few as compared with those that go to complete oxidation. In keeping with the enzyme-fatty acid concept presented above, the formation of myristic or lauric acid from palmitic acid could occur if a molecule became dissociated from the enzyme system after losing 2 or 4 carbons. One cannot, however, at present exclude the possibility that the enzymes causing interconversion may differ from those responsible for complete oxidation of the palmitic acid molecule.

¹ Jones, H. B., personal communication.

SUMMARY

1. Palmitic acids labeled with C^{14} at their carboxyl, 6th, and 11th carbons, respectively, were injected intravenously, in the form of their triglycerides, into fasted rats, and the expired CO_2 was collected at various time intervals.

2. The location of the label on the palmitic acid chain did not influence significantly the amounts of $C^{14}O_2$ expired or the rates of elimination of $C^{14}O_2$.

3. It is postulated that, once the process of breakdown of palmitic acid is initiated in the intact animal, a palmitic acid molecule is disrupted in such a manner that all of its carbons are converted to CO_2 at about the same time.

4. The implication of these findings is discussed with regard to palmitic acid oxidation and the formation of lower chain fatty acids from palmitic acid.

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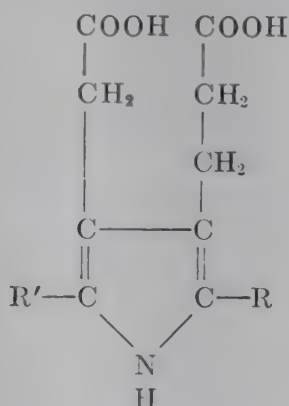
THE RÔLE OF GLYCINE IN THE BIOSYNTHESIS OF HEME*

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The nitrogen atom of glycine has been shown to be specifically utilized in the biosynthesis of heme (2, 3) but the rôle of its carbon atoms has not been fully elucidated. A reasonable working hypothesis involves the cyclization of 4 molecules of pyrroles of the following type:



whereby both naturally occurring uroporphyrin types I and III could arise. By decarboxylation of the acetic acid side chains coproporphyrin would be formed with four methyl and four propionic side chains and the latter, by decarboxylation and dehydrogenation of two of the propionic side chains at positions 2 and 4, would be converted into protoporphyrin. On this assumption one would expect to find a given carbon atom of a precursor of the pyrrole in four, or a multiple of four, positions in protoporphyrin. The only exception would be the carboxyl carbon atoms of protoporphyrin since they occur only twice in the molecule ((4), formula).

This hypothesis has been a guiding principle in the interpretation of

* Presented at the meeting of American Society of Biological Chemists at Detroit, April, 1949 (1). This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

† Research Fellow of United States Public Health Service for 1946-48. This report is part of a dissertation submitted by Norman S. Radin in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

the experimental work presented in this and the following paper (4). It is supported by the recent finding (5) that the nitrogen atom of glycine is utilized equally for Rings I and II and for Rings III and IV. In the case of nitrogen it appeared likely that 4 glycine molecules are used, and α -carbon atom entering one of the α positions of each pyrrole ring.

A study of the utilization of the carbon atoms of glycine was undertaken with red blood cells of the duck (6), either in plasma or in a buffer, as the biosynthetic medium. To evaluate the extent of the use of the carbon atoms of glycine, the experiments with C^{14} -tagged glycine were carried out in the presence of N^{15} -labeled glycine, which was included in order to determine whether synthesis had occurred and to evaluate the extent of utilization of the carbon atoms of glycine.

EXPERIMENTAL

Carboxyl-Labeled Glycine—The sodium salt of carboxyl-labeled C^{14} -acetic acid was brominated and converted to ethyl bromoacetate by the method of Auwers and Bernhardt (7). The ester was converted to glycine by the procedure of Schoenheimer and Ratner (8). The glycine activity was calculated to be 80.5 μ c. per mm from the value supplied by the Oak Ridge National Laboratory for the $BaCO_3$ used to make the sodium acetate.

$C_2H_5O_2N$. Calculated, N 18.7; found, N 18.7

Methylene-Labeled Glycine—Methylene-labeled glycine was synthesized from methyl-labeled acetic acid. Sodium acetate was converted to its anhydride by treatment with *p*-toluenesulfonyl chloride (9). The bromoacetic acid was made from the anhydride essentially by the method of Natelson and Gottfried (10) and aminated with aqueous ammonia. This procedure was developed by one of us (N. R.) in collaboration with Dr. Engelman. The activity (calculated) was 97.3 μ c. per mm.

$C_2H_5O_2N$. Calculated, C 32.0, H 6.7; found, C 32.0, H 6.5

The yield of radioactive glycine was augmented by adding inactive glycine to the mother liquors and isolating glycine as before. The same quantity of glycine was obtained with an activity a little less than one-fourth that of the main crop.

Other Compounds—Radioactive formic acid was made by catalytic reduction of potassium bicarbonate (11). The activity of the potassium formate was 208 μ c. per mm. Deuterioserine was the kind gift of Mr. D. Elwyn of this laboratory.

Glycine prepared essentially according to the directions of Schoenheimer and Ratner (8), containing 30.6 atom per cent excess N^{15} , was used

in experiments through Experiment DB17 and 31.2 atom per cent excess N^{15} was employed in later experiments.

Incubation Technique—The blood used in each group (*i.e.* Experiments DB19A, DB19B, etc.) was obtained from a duck by cutting the jugular vein. The heparinized blood was filtered through cheese-cloth and 3 mg. each of Na penicillin G and streptomycin $\cdot$ $CaCl_2$ were added to each 100 ml. of blood. A control experiment with N^{15} -glycine showed that the antibiotics did not slow the synthetic process. Incubations were carried out as previously described (6).

The blood samples used ranged from 20 to 35 ml. The isotopic compounds were added as isotonic solutions. Acids were added as sodium salts, with the exception of potassium formate. In the precursor dilution experiments, salt instead of the diluting compound was added to the control.

For the experiments with washed cells, the plasma was removed by centrifugation, and the cells were washed once or twice with normal saline and then suspended in a mixture of equal volumes of normal saline and sodium phosphate buffer (pH 7.4), together with the antibiotics and the compounds being studied. Hemolysis during the incubation period was little more than with whole blood.

Hemin Isolation—Hemin was isolated and recrystallized by the methods previously described (12).

Isotope Measuring Techniques—The N^{15} and deuterium determinations were performed with a mass spectrometer by previously described techniques (13).

The C^{14} analyses were made with a Tracerlab end window Geiger-Müller counter. Most samples were counted twice for 4096 counts, the second after rotating the sample in the sample holder. Most of the samples counted were "infinitely thick." For those which were not, appropriate corrections were made. Different correction curves were found for hemin and $BaCO_3$, in confirmation of the work by Yankwich and Weigl (14). All C^{14} values are reported on the basis of the carbon content of the compound counted; *i.e.*, the activity obtained per standard dish per minute (counts per minute) is divided by the fraction of the carbon in the compound.

RESULTS AND DISCUSSION

With glycine labeled with both N^{15} and C^{14} it is possible to calculate the utilization in the synthesis of protoporphyrin of either of the 2 carbon atoms of glycine. Since it has been demonstrated that the 4 nitrogen atoms of heme are most likely derived from glycine (5), the ratio (hemin- N^{15})/(glycine- N^{15}) is taken as the fraction of hemin formed from the labeled

glycine during the experimental period. This ratio should equal (hemin- C^{14})/(C^{14} of the labeled carbon atom of glycine) $\times 34/R$, where 34 is the number of carbon atoms in protoporphyrin and R is the number of carbon atoms of the porphyrin originating from either the carboxyl carbon atom or the α -carbon atom of glycine. R , therefore is equal to

$$R = 34 \times \frac{\text{hemin-}C^{14}}{2 \times \text{glycine-}C^{14}} \times \frac{\text{glycine-}N^{15}}{\text{hemin-}N^{15}} \quad (1)$$

The 2 occurs in the denominator since the C^{14} activities of the glycine, as determined, were the average for the 2 carbon atoms, only 1 of which was labeled. This derivation assumes that the dilutions of N^{15} and C^{14} are equal in the conversion of glycine to hemin.

Incubation Results with N^{15} and C^{14} Carboxyl-Labeled Glycine ($N^{15}H_2CH_2\cdot C^{14}OOH$)—When samples of duck blood were incubated with glycine

TABLE I

Incubation of N^{15} and C^{14} Carboxyl-Labeled Glycine ($N^{15}H_2CH_2\cdot C^{14}OOH$)

20 mg. of N^{15} -glycine and 10 mg. of C^{14} -glycine added to 30 ml. of duck blood and incubated at 37° for 24 hours.

Isotope concentrations of glycine added		Isotope concentrations of isolated hemin	
N^{15}	C^{14}	N^{15}	C^{14}
atom per cent excess	c.p.m.	atom per cent excess	c.p.m.
30.6	1.26×10^6	0.070	<1

labeled with N^{15} and with C^{14} in the carboxyl carbon atom, the newly synthesized heme, isolated as hemin, was found to contain N^{15} but no C^{14} (Table I). If 4 carbon atoms per heme originated from the carboxyl group of glycine, the hemin should have had an activity of 675 c.p.m. as calculated from Equation 1. It appears therefore that in the reactions utilizing glycine for the biosynthesis of porphyrins the carboxyl carbon atom of the amino acid is lost at some stage in the synthesis. Similar data showing that the carboxyl group of glycine is not utilized for heme synthesis have been demonstrated in the dog (15) and in the rat.¹

Incubation with N^{15} - and C^{14} -Methylene-Labeled Glycine ($H_2N^{15}C^{14}H_2\cdot COOH$)—When glycine labeled both with N^{15} and with C^{14} in the methylene carbon atom was incubated with red blood cells of the duck either in plasma or buffer, considerable radioactivity and N^{15} were found in the hemin. This is in agreement with the results of Altman *et al.* (16) who found C^{14} in hemin after feeding methylene-labeled glycine to rats. In our experiments, in which the doubly labeled glycine was employed, it is possible

¹ Radin, N. S., and Rittenberg, D., unpublished work.

from Equation 1 to calculate R , the number of α -carbon atoms utilized for the synthesis of hemin. The value of R determined in these experiments is given in the last column of Table II. Those experiments in which comparatively large amounts of glycine were used gave values considerably above 4, the number expected. The average from these experiments is 7.3, indicating that 8 α -carbon atoms are utilized. It would appear that 2 α -carbons of glycine are utilized per nitrogen atom. Apparently half of the glycine molecules incorporated lose their nitrogen and the carbon residue participates in the synthesis. That indeed some sort

TABLE II
Utilization of Methylene Group of Glycine for Heme Synthesis

Experiment No.	Blood volume	N ¹⁵ glycine	C ¹⁴ glycine	Glycine activity $\times 10^{-6}$	Hemin-C ¹⁴	Hemin-N ¹⁵	R^*
	ml.	mg.	mg.	c.p.m.	c.p.m.	atom per cent excess	
DB7	35	30.0	2.58	10.1	871	0.078	6.74
DB10C	20	30.0	2.58	8.85	1732		7.63
				8.79†	1628†	0.161	6.96†
DB10D	20	30.0	2.58	8.85	1718	0.157	7.48
				8.79†	1707†		7.49†
DB14A	23	5.19	2.29	4.94	1563	0.085	4.39
DB14B	23	15.9	2.29	4.94	831	0.107	5.68
DB14C	23	40.7	2.29	4.94	373	0.121	5.76
DB19A	23	5.19	2.29	5.24	2785	0.135	4.64
DB19B	23‡	5.19	2.29	5.24	1203	0.043	6.42
DB19C	23	40.0	2.29	5.24	668	0.145	8.14
DB19D	23‡	40.0	2.29	5.24	270	0.060	8.04

* See the text for the definition of R .

† Analyses made at a different date.

‡ Performed with washed cells.

of deamination occurs receives support from our finding of labeled ammonia in the plasma fraction after incubation. In one experiment the N¹⁵ concentration of the plasma ammonia was 0.7 per cent excess N¹⁵.

The above data were found in the *in vitro* system. If a similar experiment were performed in the intact animal, one would expect to find more than 8 α -carbon atoms of glycine utilized. The additional α -carbons participating in the synthesis would originate indirectly from glycine. It is known that α -carbon-labeled glycine in the intact animal gives rise to doubly labeled acetic acid (17) and that acetic acid participates in the synthesis of heme (18). Acetic acid formed from glycine in the intact animal would be utilized for porphyrin synthesis. Evidence will be presented later in the paper to show that a similar conversion of glycine to acetate does not occur in experiments *in vitro*.

The value of 8 found for R could result if glycine underwent extensive deamination and reamination. If the glycine were then reaminated by non-isotopic ammonia, the N^{15} concentration of the glycine would be decreased before it was utilized for heme synthesis. That this is not the case is shown in Experiment DB19C (Table III) in which glycine was isolated with the aid of carrier glycine from the plasma after incubation. The $C^{14}:N^{15}$ ratios of the original glycine and the glycine isolated as such and as hippuric acid were compared. The data in Table III demonstrate that the $C^{14}:N^{15}$ ratios differed only slightly; moreover, the dilution of N^{15} was only 9 per cent more than that of the C^{14} , which is too low to explain the value of R found.

Additional evidence indicating that exchange of nitrogen cannot account for the fact that R is greater than 4 is given by Experiment DB19

TABLE III

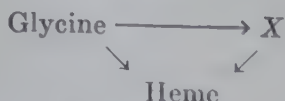
Comparison of Isotope Concentrations of Glycine Added to Blood and Glycine Isolated from Blood after 24 Hour Incubation

The glycine was isolated with aid of carrier glycine from Experiment DB19C, Table II.

Compound	Isotope concentrations		Ratio $C^{14}:N^{15}$
	C^{14}	N^{15}	
	<i>c.p.m.</i>	<i>atom per cent excess</i>	
Glycine added.....	283,000	29.5	9,610
“ isolated.....	4,090	0.402	10,160
“ “ (as hippuric acid).....	4,180	0.392	10,650

(Table II). The value of R was greater when more glycine was added. If the nitrogen exchange were occurring and if the rate were independent of the amount of glycine added, then R should decrease with increased amounts of glycine. On the other hand, if the nitrogen exchange were proportional to the amount of glycine present, the value of R should be independent of the glycine level.

It would appear that 4 α -carbon atoms of glycine enter the porphyrin molecule attached to the nitrogen atom and 4 α -carbon atoms of glycine enter the porphyrin molecule through some other intermediate as shown in the following diagram.



Thus, glycine is converted to X , and both compounds are utilized for the biosynthesis of heme. Of the two, only glycine contributes the nitrogen

atoms. Since increasing amounts of added glycine will raise the isotope concentration of the total available glycine, the C^{14} concentration in X , which we assume is derived from glycine, should increase with increasing amounts of added glycine. One would then expect the direct glycine utilization to contribute four to the value of R , independently of the amount of added glycine, but the contribution to R by the secondary utilization should increase with increased glycine. The finding of a value close to 8 at high glycine levels suggests that the amount of X derived from glycine increases with increased amounts of glycine and that the addition of 30 to 40 mg. of isotopic glycine results not only in minimizing

TABLE IV

Influence of Some Compounds on Utilization of Methylene Carbon Atom of Glycine for Heme Synthesis

Experiment No.	Blood volume	N^{15} -glycine	C^{14} -glycine	Glycine activity $\times 10^{-6}$	Diluent	Hemin- C^{14}	Hemin- N^{15}	R
	ml.	mg.	mg.			c.p.m.	atom per cent excess	
DB19E*	23	40	0.0		None	433	0.164	
DB19G*	23	40	0.0		10 mg. iminodiacetic acid	437	0.168	
DB19H*	23	40	0.0		30 mg. iminodiacetic acid	437	0.167	
DB19I	23	40	2.29	5.46	31 mg. glycolic acid	636	0.137	7.89
DB19J	23	40	2.58	5.46	None	735	0.145	7.63
DB23A	25	24.4	1.94	5.41	"	735	0.124	7.31
DB23B	25	24.4	1.94	5.41	20 mg. L-serine	702	0.115	7.53
DB23C	25	24.4	1.94	5.41	31 " Na pyruvate	759	0.121	7.74
DB23D	25	24.4	1.94	5.41	24 " " acetate	769	0.119	7.97

* 0.6 mg. of $CH_3C^{14}OOH$ added.

the dilution of the isotope by endogenous glycine but also by endogenous X . Certain compounds suggest themselves as possibly being X . Glyoxylic acid arising from glycine may be utilized directly or by condensation with glycine to form a Schiff base, $HOOCH_2-N=CH-COOH$, which on reduction could yield iminodiacetic acid, $HOOCH_2-NH-CH_2-COOH$. These two possibilities were tested. In Experiment DB19I (Table IV) glycolic acid was incubated with blood together with N^{15} and α -carbon C^{14} -labeled glycine. If the glycolic acid were oxidized to glyoxylic acid and the latter in turn utilized for heme synthesis, the value of R would be lowered. The comparison with control Experiments DB19C, DB19D, and DB19J (Tables II and IV) showed no effect. Similarly iminodiacetic acid was tested as a possible intermediate by incubating a non-isotopic

sample of this compound with red blood cells along with N^{15} -glycine. If the iminodiacetic acid were an intermediate, the N^{15} concentration of the resulting hemin should be lower than the control. In Experiments DB19E, DB19G, and DB19H (Table IV) it can be seen that the N^{15} concentrations of the hemin samples were the same. That iminodiacetate does not inhibit the rate of heme synthesis was tested by the addition of carboxyl-labeled acetic acid. Not only were the N^{15} concentrations the same but also the radioactivities were equal. Iminodiacetic acid does not appear to be an intermediate in heme synthesis.

It is known that glycine may give rise to serine (19–21), pyruvic acid (22), and acetic acid (17), and that the α -carbon of glycine may yield formic acid (21). To test these possible conversion products as intermediates two types of experiments were performed. Glycine labeled with N^{15} and C^{14} in the α -carbon atom was incubated either with non-isotopic serine, pyruvate, or acetate. It can be seen in Table IV, Experiment DB23, that these substances affected neither the isotope concentration nor the value of R . This *in vitro* system seems to be incapable of forming acetic acid from glycine in contrast to the ready conversion *in vivo*.

It has been suggested that the α -carbon atom of glycine is converted to formic acid and the possibility exists that the secondary utilization route for glycine is via formic acid. This possibility was tested by incubation of a blood sample with C^{14} -formic acid together with N^{15} -glycine. It was found that, whereas normal synthesis took place as indicated by the hemin- N^{15} concentration (0.151 atom per cent excess N^{15}), very little radioactivity was found (4.2 c.p.m.). Similar results are reported by Bufton, Bentley, and Rimington (23).

The negative results obtained in the precursor dilution experiments above must be interpreted with caution. It is possible that the actual intermediates are not the compounds tested but are "active" forms which are not readily produced in this system. The additional possibility exists that the compounds tested could not penetrate the cell membranes at a sufficiently high rate.

It is to be noted that in Experiment DB19 (Table II) the washed cells were not as active as the cells in plasma, but in Experiments DB19C and DB19D, with high levels of glycine, the R values are the same. In Experiments DB19A and DB19B, with low levels of glycine, the washed cell preparation gave a higher R value than the control blood sample. This is in agreement with the previous suggestion that in whole blood there exists a non-nitrogenous derivative of glycine and that this can be utilized for the synthesis of heme.

SUMMARY

With glycine labeled with N^{15} and with C^{14} in either the carboxyl carbon atom or α -carbon atom the following has been found.

1. The carboxyl carbon atom of glycine is not utilized for heme synthesis.

2. 8 α -carbon atoms of glycine seem to be utilized for the synthesis of the porphyrin molecule; for each nitrogen atom 2 α -carbon atoms of glycine are involved.

3. Glycolic acid, iminodiacetic acid, pyruvic acid, serine, acetic acid, and formic acid are probably not involved in the utilization of glycine for heme formation.

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THE RÔLE OF ACETIC ACID IN THE BIOSYNTHESIS OF HEME*

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In the preceding paper evidence was presented which suggested that 8 carbon atoms of protoporphyrin are derived from the α -carbon atom of glycine (2). The synthesis of the porphyrin molecule, which contains 34 carbon atoms, must involve the participation of other compounds.

It has previously been shown that administration of deuterioacetate (CD_3COOH) to rats results in the formation of deuteriohemin (3). The methyl group of acetate, therefore, is utilized either for methine carbon atoms or for carbon atoms of the side chains since these are the only carbon atoms bonded to hydrogen. In the present study the rôle of acetic acid in the synthesis of heme was further investigated by means of the blood system of the duck *in vitro* (4). Carboxyl and methyl C^{14} -labeled acetic acid, pyruvic acid labeled with C^{14} in the α position, acetone labeled with C^{14} in the methyl group, and C^{14}O_2 were employed. The possible participation of certain members of the tricarboxylic acid cycle in heme synthesis was tested by the precursor dilution technique. In most experiments N^{15} -labeled glycine was included in order to determine the amount of synthesis and to permit a comparison of the relative utilization of various compounds for heme synthesis.

It was found that both carbon atoms of acetate as well as the α -carbon atom of pyruvic acid are utilized for heme synthesis but that acetone and CO_2 are not utilized in the *in vitro* system.

EXPERIMENTAL

Carboxyl-Labeled Acetic Acid—Acetic acid was prepared by the addition of radioactive carbon dioxide to methyl magnesium iodide. The C^{14}O_2 was generated from $\text{BaC}^{14}\text{O}_3$ obtained from the Atomic Energy Commis-

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sion. The radioactive material was diluted with normal BaCO_3 to give an activity of 199 $\mu\text{c. per mm.}$

Other Compounds—Methyl-labeled sodium acetate was the gift of Dr. M. Engelman of this laboratory. Its activity was 97.3 $\mu\text{c. per mm.}$ Methyl-labeled C^{14} -acetone with an activity of 65 $\mu\text{c. per mm.}$ was prepared in this laboratory by Mr. T. Duane Price. α -Labeled C^{14} -pyruvic acid (activity of 10 $\mu\text{c. per mm.}$) was prepared from pyruvamide kindly furnished by Dr. H. S. Anker of the University of Chicago. The pyruvamide was hydrolyzed by heating with hydrochloric acid (5).

Incubation—The incubation, isolation, and isotope analysis techniques have been described (2, 4). Whenever silver or barium salts were measured suitable corrections for back-scattering (6) were made.

TABLE I

C^{14} Activity in Hemin Made from Carboxyl-Labeled Acetate ($\text{CH}_3\text{C}^{14}\text{OOH}$)
The activity of carboxyl carbon of acetic acid is 3.61×10^5 c.p.m.

Blood sample	Acetate added		Hemin- N^{15}	C^{14} activity of hemin	
	Normal NaAc	C^{14} -acetate		Found	Calculated*
	mg.	mg.	atom per cent excess	c.p.m.	c.p.m.
A	0	2.4		1755	1755
B	5.3	2.4		1310	4200
C	10.3	2.4		1110	5900
D	19.5	2.4	0.139	852	7800
E	50.4	2.4	0.142	345	7600
F	98.7	2.4	0.138	146	6150

* Calculated value = found value $\times$ (total amount of acetate)/(amount of radioactive acetate).

Incubation with Carboxyl-Labeled Acetic Acid ($\text{CH}_3\text{C}^{14}\text{OOH}$)—Six 26 ml. samples of blood, from the same duck, were incubated for 24 hours with glycine and various amounts of acetic acid as indicated in Table I. To each blood sample 6 ml. of water were added and appropriate amounts of sodium chloride to maintain isotonicity. The same amount of radioactive carboxyl-labeled sodium acetate (2.4 mg.) was added to each blood sample and the total acetate concentration varied by additions of normal acetate. 50 mg. samples of N^{15} -glycine (30.6 atom per cent excess N^{15}) were added to Samples D, E, and F, and 50 mg. samples of non-isotopic glycine were added to Samples A, B, and C.

In order to compare the activities of the different hemin samples isolated, the observed values for activity were multiplied by the factor (total amount of acetate)/(amount of radioactive acetate) which corrects for the dilution caused by the addition of non-radioactive acetate. The results

are given in Table I. It can be seen that the activity of the hemin reached a maximum when about 22 mg. of acetate were employed (Sample D); addition of larger amounts of acetate did not increase the activity. It is to be noted that the amount of heme synthesized as indicated by the N^{15} concentration was the same in Samples D, E, and F. Since heme can be formed in this system without addition of acetate the blood must contain either acetate or potential sources of acetate. The labeled acetate added was therefore diluted not only by the added normal acetate but by the endogenous acetate. This accounts for the increasing calculated activity of the heme as the amount of added acetate was increased. In Samples D, E, and F, in which relatively large amounts of acetate were added, the dilution due to endogenous acetate appears to have become negligible; the calculated activity of the hemin reaches a maximum value.

TABLE II

Comparison of Synthesis in Whole Blood and Washed Cells

0.6 mg. of $CH_3C^{14}OOH$ (1.3×10^7 c.p.m.) and 40 mg. of N^{15} -labeled glycine (30.6 atom per cent excess) were added to each sample (23 ml.).

Blood volume sample ml.	Isotope concentration	
	N^{15} atom per cent excess	C^{14} c.p.m.
Whole blood.....	0.164	433
Washed cells.....	0.078	525

In another experiment the utilization of acetate $CH_3C^{14}OOH$ for heme synthesis was compared in whole blood and in washed red cells. It can be seen in Table II that glycine utilization is lowered, as previously found (2, 4), whereas acetate utilization was considerably enhanced by the removal of the plasma. It seems that, although the synthetic capacity was lowered, the dilution of the C^{14} -acetic acid by potential acetic acid is much lower in washed cells than in whole blood.

Degradation of Hemin Derived from Carboxyl-Labeled Acetate—The symmetry of the porphyrins indicates that a given carbon atom of a precursor should be found four, or a multiple of four, times in each molecule. The two carboxyl groups of heme constitute an exception.

To determine the distribution of the C^{14} in the hemin produced from carboxyl-labeled acetate, the radioactive hemin was decarboxylated. Hemin samples obtained from duck blood incubated with carboxyl-labeled acetate were heated to 380° , purified hydrogen was passed through the flask, and the carbon dioxide liberated (20 to 30 per cent yield) was washed with sulfuric acid and toluene and absorbed in barium hydroxide. The

activity of the precipitate was compared to that of hemin. The results of the experiments are given in Table III.

TABLE III
Ratio of Activities of Carboxyl Group of Hemin to That of Hemin

Experiment No.	Blood volume	Sodium acetate*	Hemin-N ¹⁵	Hemin-C ¹⁴	Carboxyl group of hemin-C ¹⁴	COOH-C ¹⁴ / Hemin-C ¹⁴
	ml.	mg.	atom per cent excess	c.p.m.	c.p.m.	
DB20A†	80‡	2.16	0.087	421	4420	10.5
DB21	200‡	28.0		184	1797	9.8
DB24C				402	3320	8.3
DB25				158	1286	8.0
DB20D†	80‡	2.16§	0.088	308	4240	13.8

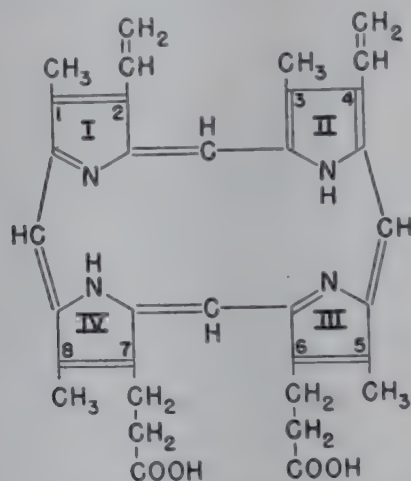
Experiments DB24C and DB25 were performed with pooled samples of hemin obtained from experiments with carboxyl-labeled acetate. The decarboxylation of hemin in Experiment DB24C was carried out with mineral oil as a suspension medium for the hemin.

* Sodium acetate activity was 1.3×10^7 c.p.m.

† Added 142 mg. of glycine containing 31.2 atom per cent excess N¹⁵.

‡ Washed cells used.

§ 90 mg. of sodium pyruvate added.



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FIG. 1

If all the activity were in the two carboxyl groups, the CO₂ obtained from them should have 34/2 or 17 times the activity of the hemin. In none of these experiments is this value reached. The average, exclusive of the one experiment in which pyruvic acid was added, on the activities of the carboxyl carbon atoms relative to those of the total heme is 9.2.

More than half (9/17), but not all of the activity is in the COOH carbon atoms. It is evident that other carbon atoms of heme are derived from the carboxyl group of acetate.

This conclusion was confirmed by degrading a sample of radioactive hemin from Experiment DB21 (Table III) according to the method used by Wittenberg and Shemin (7). Two pyrrolines are obtained, methylmethoxyethylmaleimide from Rings I and II, and hematinic acid from Rings III and IV (Fig. 1). In these pyrrolines the carbon atoms of the side chains are maintained, but the methene carbon atoms are removed. 1 additional carbon atom is introduced, as a methoxy group on the side chain of methoxyethylmaleimide. The data are given in Table IV.

TABLE IV

Distribution of C¹⁴ in Pyrrole Rings of Heme Formed from Carboxyl-Labeled Acetate (Experiment DB21)

Compound	Activity	Observed relative activity	Calculated relative activity
Hemin	198*		1
	184†		1
Methylmethoxyethylmaleimide (Rings I and II)	82.4*	0.42	
	78.9†	0.43	0.45
Hematinic acid (Rings III and IV)	299*	1.51	
	301†	1.64	1.67
Ammonium hematinate	316†	1.72	1.67

The activities followed by the asterisk (*) were determined at one time; those with dagger (†) at another.

On the assumption that only 4 additional atoms, 1 in each pyrrole unit, contain activity their relative activities can be calculated on the basis of unity for the values observed for hemin. As the relative activity of the carboxyl groups in Experiment DB21 is 9.8 the postulated 4 additional active atoms would have relative activities of 3.6, as calculated from the expression

$$1 = \frac{2 \times 9.8 + 4x}{34}$$

in which x is the average relative activity of the 4 labeled carbon atoms other than the carboxyl groups.

On the above assumption, the methylmethoxymethylmaleimide, having 8 carbon atoms, should have a relative activity one-eighth of that of the

labeled atom, *i.e.*, $3.6/8 = 0.45$. The hematinic acid, which also contains the labeled carboxyl group, should have a relative activity equal to one-eighth of the sum of the 2 labeled atoms, *i.e.* $(9.8 + 3.6)/8 = 1.67$. The data are in satisfactory agreement (see Table IV) with this hypothesis. They show that besides the carboxyl groups each pyrrole unit contains 1 or more carbon atoms of lower activity derived from the carboxyl group of acetate. Further, the methene carbon atoms of heme probably are not derived from the carboxyl group of acetic acid, since practically all the activity is found in the fragments isolated.

TABLE V

Relative Utilization of Methyl and Carboxyl Groups of Acetic Acid for Heme Synthesis

Experiment No.	Blood volume	C ¹⁴ -sodium acetate	Acetate activity $\times 10^{-5}$	Position labeled	Hemin-N ¹⁵	Hemin-C ¹⁴	S*
	ml.	mg.	c.p.m.		atom per cent excess	c.p.m.	
DB10B	20	0.6	88.7	CH ₃	0.165	2677	6.0
DB10A	20	0.6	180	COOH	0.165	910	
DB12B	21	20.0	2.44	CH ₃	0.113	556	6.1
DB12C	21	20.0	6.03	COOH	0.113	227	
DB22A	21.5	0.708	27.0	CH ₃	0.083	716	5.9†
DB22C	21.5	0.60	116	COOH	0.082	444	
DB22B‡	21.5‡§	0.708	27.0	CH ₃	0.083	608	7.4†
DB22D‡	21.5‡§	0.60	116	COOH	0.085	297	

In all experiments 25 mg. of N¹⁵-labeled glycine were added except in Experiments DB12B and DB12C in which 30 mg. of the labeled glycine were added.

* See the text for definition of S.

† The ratio has been corrected for the difference in the amounts of acetate added, assuming that the hemin activity is directly proportional, at this level of acetate, to the amount of acetate added.

‡ Washed cells.

§ These samples also contained 25 mg. of sodium pyruvate.

This suggests that acetate is utilized by two or more routes. One route results in formation of the heme carboxyl groups; the other, on the basis of the generic pyrrole hypothesis, may be expected to furnish 4, or a multiple of 4, atoms to each heme molecule; that is 1 or more per pyrrole group. However, the latter carbon atoms enter at a lower level of activity.

Methyl-Labeled Acetic Acid—In an attempt to estimate the number of carbon atoms supplied by the methyl group of acetic acid, carboxyl- and methyl-labeled acetic acids were compared in identical blood samples. The results of these experiments are given in Table V. Glycine containing N¹⁵ was also added to the incubation mixtures. It will be noted that in

comparable experiments the N^{15} concentrations of the hemin samples were the same, indicating that the rate of hemin synthesis was also the same. The ratios in the last column (S) in Table V represent the relative utilization of the methyl group of acetate as compared to the carboxyl group. They are calculated by dividing the dilution of isotope observed with methyl-labeled acetate by the dilution found with carboxyl-labeled acetate. That is

$$S = \frac{\frac{(\text{Activity of hemin from methyl-labeled acetate})/(\text{activity of methyl-labeled acetate})}{(\text{Activity of hemin from carboxyl-labeled acetate})/(\text{activity of carboxyl-labeled acetate})}}$$

The average value of S in experiments in which only acetate was used was 6.0. Since we have taken as the standard of relative activity hemin derived from carboxyl-labeled acetate, the relative activity of hemin derived from methyl-labeled acetate is 6. The ratio appears to be the same over a wide range of acetate levels, and washing the cells (Experiment DB22, Table V) has no effect. Addition of normal pyruvate increases the value of S ; *i.e.*, pyruvate reduces the activity of the hemin made from carboxyl-labeled acetate more than that made from methyl-labeled acetate.

That more methyl groups than carboxyl groups are used for the biosynthesis of heme agrees with the hypothesis that a generic pyrrole is involved in porphyrin synthesis. If the tetrapyrrole compound first formed is an octacarboxylic acid (uroporphyrin) in which the carboxylic acid groups are derived from the COOH group of acetic acid and the adjacent methylene groups are derived from the methyl group of acetic acid, then the subsequent decarboxylations to yield protoporphyrin would result in a ratio of 4 for the relative utilization of the methyl and carboxyl groups of acetic acid for this part of the molecule. This in part may explain the observed value of 6.

The ratio indicates that considerable decarboxylation must be involved in the synthesis of heme. This is supported by analysis of the plasma CO_2 in Experiments DB12B and DB12C after the incubation period. The plasma was acidified and the carbon dioxide aerated into baryta. The $BaCO_3$ samples, about 11 mg. each, had activities of 4.28×10^4 (from Experiment DB12B) and 1.72×10^5 (from Experiment DB12C). These represent dilutions of 7- and 4-fold, respectively. The finding of high activity in the bicarbonate of Experiments DB12B and DB12C (Table V) indicates that duck blood can effect the complete oxidation of acetate to CO_2 . The finding that bicarbonate obtained from carboxyl-labeled acetate was relatively more active than that from the methyl-

labeled acetate shows that decarboxylation as well as complete oxidation is occurring in this system.

Degradation of Hemin Prepared from Methyl-Labeled Acetate—In order to ascertain some of the positions in hemin derived from the methyl carbon of acetate, the hemin prepared from methyl-labeled acetate was subjected to oxidation by chromate-sulfuric mixture according to a slightly modified procedure for determining methyl groups (8). Under the conditions of this reaction methyl groups and the carbon atoms attached to them are oxidized to acetic acid. 112 mg. of hemin (0.17 mm) were refluxed for 2.5 hours with a mixture of sulfuric acid (5 ml.) and chromic acid (20 ml. of a solution of 21 gm. of CrO_3 in 125 ml. of water). Excess chromic acid was reduced with hydrazine, the solution was steam-dis-

TABLE VI
Distribution of C^{14} in Hemin

Distribution of C^{14} in acetic acid obtained by oxidation of hemin made from methyl-labeled acetic acid ($\text{C}^{14}\text{H}_3\text{COOH}$).

Compound	C^{14}	Position in porphyrin	Relative activity*
	<i>c.p.m.</i>		
Isolated hemin.....	1213		6.0
Acetic acid.....	1570	Methyl side chains and β -carbon atoms in pyrroles in positions 1, 3, 5, and 8	7.8
Carboxyl group of acetate....	1300	β -Carbon atoms in pyrroles in positions 1, 3, 5, and 8	6.4
Methyl group of acetate.....	1840†	Methyl side chains	9.1

* The relative activity of the hemin is taken as 6.0.

† Calculated.

tilled, and the acetic acid (yield 0.66 mm) was converted to the silver salt. The ground, dried silver acetate, after being analyzed for activity, was decarboxylated. The silver salt was suspended in boiling carbon tetrachloride and an excess of a dilute solution of bromine in carbon tetrachloride was added (9). The evolved carbon dioxide was collected in barium hydroxide. In a test experiment with methyl-labeled silver acetate, the carbon dioxide evolved had no activity; the carbon dioxide is derived exclusively from the carboxyl group. The activities of the hemin, acetate, methyl carbon atom, and carboxyl carbon atom of acetate are given in Table VI. The activity in the COOH group of the acetic acid is that of the β -carbon atoms of the pyrroles of hemin in positions 1, 3, 5, and 8 (see Fig. 1); the value for the acetic acid is the average activity of these carbon atoms and the attached methyl groups. The activity of the

methyl groups attached to positions 1, 3, 5, and 8 is calculated from the above data. These calculations are made on the assumption that all four pyrrole rings are labeled equally. On this basis the data show that the β -carbon atoms in positions 1, 3, 5, and 8 of the pyrroles are derived from the methyl group of acetic acid, as are the methyl side chains of hemin, and that a minimum of 8 carbon atoms of heme is derived from the methyl group of acetic acid.

Another sample of hemin was decarboxylated. The carboxyl carbon atoms of the propionic acid side chains were almost inactive (activity of carboxyl carbon was one-seventh of that of the hemin).

Effect of Pyruvic Acid on Heme Synthesis—The rôle of pyruvic acid in the biosynthesis of heme was investigated in view of its known close relationship to acetic acid metabolism and the possibility that it might be directly involved as a precursor.

TABLE VII
C¹⁴ Concentration in Hemin

Incubation with α -C¹⁴-labeled pyruvate, CH₃C¹⁴OCOOH (10 μ c. per mM), and N¹⁵-labeled glycine in presence and absence of non-isotopic acetate.

Experiment No.	Compounds added			Isotope concentration of hemin	
	Pyruvate	Glycine	Non-isotopic acetate	N ¹⁵	C ¹⁴
	mg.	mg.	mg.	atom per cent excess	c.p.m.
DB11A	8.5	25	0	0.150	57
DB11B	8.5	25	25	0.144	28

Two blood samples were incubated, each containing 8.5 mg. of α -C¹⁴-pyruvic acid (ammonium salt) and 25 mg. of N¹⁵-glycine to serve as a control on the rate of heme synthesis. The second sample contained an additional 25 mg. of normal sodium acetate. The results are given in Table VII. It is apparent from these data that pyruvic acid can serve as a precursor of heme. While the N¹⁵ concentrations in both hemin samples were the same, demonstrating the same rate of synthesis of heme, the sample containing the normal acetate had about half the activity.

In other experiments, radioactive acetic acid was incubated with blood, with or without normal pyruvic acid. The results are summarized in Table VIII. In every case (except Experiment DB16), the presence of pyruvic acid resulted in a lowered hemin-C¹⁴ concentration, although the heme syntheses in comparable experiments, as indicated by N¹⁵ concentrations, were equal. Even with the highest ratios of pyruvate to acetate used the decrease was no greater than 33 per cent. It is apparent that a

small amount of labeled acetate cannot be diluted by a relatively large amount of unlabeled pyruvate. This indicates that in the utilization of acetic acid its conversion to pyruvic acid is not obligatory.

In Experiment DB22 (Table VIII) it was found that the depression of the activity of the hemin made from CH_3 -labeled acetate was only about half that of the hemin made from COOH -labeled acetate. Further in Experiment DB20 (Table VIII) it was observed that, while the addition of pyruvic acid had no effect on the N^{15} concentration of the heme formed, it reduced the incorporation of C^{14} from the carboxyl group of acetate by about 25 per cent. Despite the lowering of the activity of the total mole-

TABLE VIII
Effect of Non-Isotopic Sodium Pyruvate on C^{14} -Acetate Utilization

Experiment No.	Sodium acetate	Label position	Blood volume	Sodium pyruvate	Hemin- N^{15}	Hemin- C^{14}
	mg.		ml.	mg.	atom per cent excess	c.p.m.
DB10A	0.6	COOH	20	0	0.165	911
DB10E	0.6	"	20	25	0.172	705
DB16A	19.6	CH_3	26	0		577
DB16D	19.6	"	26	6.7		582
DB17A	20.5	COOH	26.5	0	0.087	138
DB17D	20.5	"	26.5	6.6	0.090	113
DB20A*	2.16	"	80	0	0.087	421
DB20D*	2.16	"	80	90	0.088	308
DB22A*	0.708	CH_3	22	0	0.083	716
DB22B*	0.708	"	22	25	0.083	608
DB22C*	0.6	COOH	22	0	0.082	444
DB22D*	0.6	"	22	25	0.085	297

* Experiments DB20 and DB22 were performed with washed cells.

cule, the activity of the carboxyl groups of the hemin was unchanged (Table III). The pyruvic acid had diluted the carboxyl carbon atoms of acetate which were the source of heme carbon atoms other than its carboxyl groups. Pyruvate cannot be used for heme synthesis solely by virtue of its conversion to acetate, for the same dilution of the carboxyl groups of the hemin as of the hemin should have been observed. It is clear that the ratio of the activities of the carboxyl group of heme to that of the heme will be higher when pyruvate is present. This dilution, caused by pyruvate, explains, in part at least, the fact that the carboxyl groups of acetate appear in the hemin at two levels of activity.

Further Studies of Acetate Utilization—Some experiments, carried out by the dilution technique with COOH -labeled acetate, were performed in an effort to uncover intermediates derived from acetic acid. The data

from these experiments are given in Table IX. Some of the compounds involved in the Krebs cycle were tested, since it is well established that acetic acid can enter the cycle. Considering the precision of the technique used, the data in Table IX are inconclusive except with large amounts of ketoglutaric acid (Experiment DB20). The reductions in activity observed with ketoglutaric acid may be due to conversion to pyruvic acid, which has already been shown to exert this effect.

TABLE IX

Utilization of $\text{CH}_2\text{C}^{14}\text{OOH}$ in Presence of Other Non-Isotopic Compounds

Experiment No.	Sodium acetate	Label position	Blood volume	Diluent	Hemin- C^{14}	Hemin- N^{15}
	mg.		ml.		c.p.m.	atom per cent excess
DB12	20.0	COOH	21	None	227	0.113
				10.4 mg. succinic acid	222	0.120
				10.0 " oxalacetic acid	208	0.113
				7.0 " butyric acid	192	0.099
DB16	19.6	CH_3	26	None	577	
				8.8 mg. ketoglutaric acid	591	
				7.1 " succinic acid	600	
DB17	20.5	COOH	26.5	None	138	0.087
				8.9 mg. ketoglutaric acid	135	0.098
				7.2 " succinic acid	130	0.094
DB20A*	2.16	"	80	None	421	0.087
DB20G*	0.6	"	22	38.4 mg. ketoglutaric acid	297	0.116

* Experiment DB20 was performed with washed cells.

Experiments with Other Labeled Compounds

Acetone—Two blood samples were incubated with 30 mg. of N^{15} -glycine and 0.2 mg. of C^{14} -methyl-labeled acetone (activity 65 $\mu\text{c.}$ per mm). Although the hemin samples contained 0.106 per cent excess N^{15} , they were entirely devoid of C^{14} activity.

Pyruvamide—5.7 mg. of $\alpha\text{-C}^{14}$ -labeled pyruvamide were incubated with a blood sample in the usual way and no utilization was found. This difference between pyruvamide and pyruvic acid is consonant with that found in rats (10).

Carbon Dioxide—0.0126 mm (41.1 $\mu\text{c.}$) of $\text{NaHC}^{14}\text{O}_3$ was added to 23 ml. of blood, together with N^{15} -glycine. The extremely active bicarbonate resulted in hemin with an activity of only 13 c.p.m. Apparently carbon dioxide fixation represents, in duck blood, a negligibly small fraction of porphyrin carbon requirements (11). The carbon dioxide recovered from the plasma at the end of the experiment had an activity of 3300 c.p.m.

DISCUSSION

The experiments demonstrate that both carbon atoms of acetate are used for the biosynthesis of protoporphyrin. The locations of some of the carbon atoms of the porphyrin which are derived from the carbon atoms of the acetate were ascertained by degradation.

The carboxyl carbon atoms of heme are derived from the carboxyl carbon atom of acetate. The carboxyl groups of hemin secured in the experiment with carboxyl-labeled acetate had activity of 9.8 relative to that of the total porphyrin. There probably are also at least 4 other carbon atoms of the porphyrin which are derived in part from the carboxyl carbon atoms of acetate. These carbon atoms would have a maximum relative activity of 3.6. This suggests that these carbon atoms of the heme may in part originate from another compound or from the methyl group of acetate.

The four methyl group side chains are derived from the methyl group of acetate, as are the 4 β -carbon atoms of the pyrrole rings in positions 1, 3, 5, and 8. The methyl groups in the hemin obtained in the experiment with methyl-labeled acetate had an activity 9.1 times as great as that of the total carbon in the hemin obtained from carboxyl-labeled acetate; the β -carbon atoms had a relative activity of 6.4; the hemin a relative activity of 6. The 8 carbon atoms in the hemin obtained from methyl-labeled acetate had a total activity of 62 (4×9.1) + (4×6.4). The total activity of the hemin is 204 (34×6). More than two-thirds of the total activity in the hemin ($(204 - 62)/204$) is therefore unaccounted for by the isolated fragments. It is clear that many other positions in the hemin are derived from the methyl group of acetate.

If we assume that the activity of the methyl side chains is representative of the activity of the other carbon atoms derived from the methyl group of acetate, then there must be about 16 ($142/9.1$) of these. These calculations, together with contribution of the α -carbon atom of glycine, account for most of the carbon atoms of protoporphyrin.

Of the 34 carbon atoms of heme, 8 may be derived from the α -carbon atom of glycine. 10 of the remaining 26 carbon atoms have been obtained by degradation, the two carboxyl groups, the four methyl side chains, and the 4 β -carbon atoms of the pyrroles in positions 1, 3, 5, and 8. It is reasonable to assume that the 2 methylene carbon atoms adjacent to the carboxyl groups also arise from the methyl group of acetate.

Of the remaining 14 carbon atoms it would appear that at least 4 are in part derived from the carboxyl carbon atom of acetate. It would appear that some positions in the heme molecule are derived from both carbon atoms of acetate.

Pyruvate is not utilized for heme synthesis by way of acetate; addition

of non-isotopic pyruvic acid to a system containing carboxyl-labeled acetate lowers the activity of the total carbon atoms in the heme but not of the carboxyl groups. This suggests that part of the utilization of acetate may proceed through pyruvate or a compound formed from pyruvate. Indeed, α -labeled pyruvate is utilized for heme synthesis.

The inference, drawn from the data in this paper, that the carboxyl carbon atoms of acetate are not used for the formation of the methine bridges, is supported by the finding of Wittenberg and Shemin (12) that the α -carbon atoms of glycine supply the 4 methine bridge carbon atoms.

SUMMARY

1. Both the carboxyl and the methyl groups of acetate are used for heme synthesis.

2. The carboxyl group of acetate is a source of the two carboxyl groups of heme. Also, it contributes to at least 4 of the carbon atoms in the porphyrin molecule. These carbon atoms have a lower activity than the carboxyl carbon atoms.

3. Hemin produced from methyl-labeled acetate is 6 times as radioactive as that formed from carboxyl-labeled acetate of the same activity. It has been shown that the methyl carbon is converted to the methyl and β -carbon atoms of the pyrrole as well as to other unidentified positions.

4. Pyruvate is utilized for synthesis of heme; acetone and CO_2 are not.

5. The data suggest that most or all of the carbon atoms of heme are derived from acetate and glycine.

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FATTY ACID SYNTHESIS BY ENZYME PREPARATIONS OF *CLOSTRIDIUM KLUYVERI*

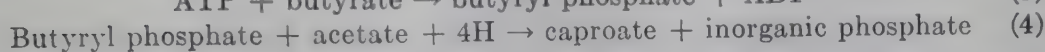
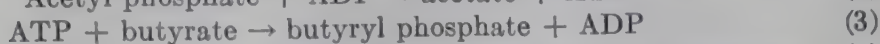
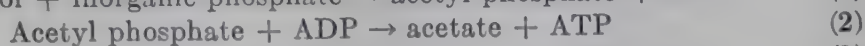
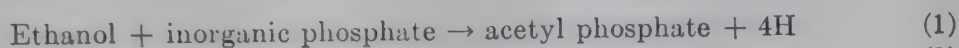
VI. REACTIONS OF ACYL PHOSPHATES

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The conversion of ethyl alcohol and butyrate to caproate by *Clostridium kluyveri* was postulated (1) to involve the following reactions.¹



Reaction 1 was observed in cell-free enzyme preparations of *C. kluyveri* (21), but the participation of the remaining reactions is supported only by indirect evidence obtained from studies on other bacteria (2). Koepsell *et al.* (12) demonstrated the formation of butyryl phosphate from acetyl phosphate and butyrate by cell-free extracts of *Clostridium butylicum*, and Lipmann (13, 14) demonstrated the reversible transfer of phosphoryl groups between acetyl phosphate and the adenylic acid system by enzyme preparations of *C. butylicum* and *Lactobacillus delbrueckii*, and further postulated that the transfer of the phosphoryl group between acetyl phosphate and butyrate is mediated by adenosine pyrophosphate.

In order to obtain a clearer insight into the mechanisms of fatty acid synthesis we have investigated the occurrence of Reactions 2, 3, and 4 and also several other reactions of acyl phosphates in enzyme preparations of *C. kluyveri*.

Methods

Chromatographic Analysis of Acyl Phosphates

To obtain a quantitative estimation of the various acyl phosphates in a mixture, these substances were converted to their hydroxamic acid derivatives (17), which are easily separated by paper chromatography. After separation, the various hydroxamic acids were located on the paper chromatogram by converting them to their highly colored ferric iron complexes; these were readily extracted from the paper and estimated

¹ The following abbreviations are used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; AMP, adenosine monophosphate.

quantitatively by a colorimetric procedure. Details of the method are as follows:

Reagents—

Hydroxylamine hydrochloride. A 28 per cent solution of hydroxylamine hydrochloride was mixed with an equal volume of 3.5 N sodium hydroxide just prior to use.

Ferric chloride. Two different solutions were used, a strong solution, made by dissolving 50 gm. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 1 liter of 95 per cent alcoholic 0.1 N HCl and a weak solution made by mixing 5 gm. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 25 ml. of concentrated HCl, and 575 ml. of water.

Ethanol, 95 per cent and absolute.

*Procedure—*From 0.5 to 1.0 ml. of the test solution was added to 0.5 ml. of the hydroxylamine reagent. After 10 minutes at room tempera-

TABLE I

R_F Values for Hydroxamic Acid Derivatives of Acyl Phosphates

Hydroxamic acid derivative	<i>R_F</i> value*
Acetyl phosphate.....	0.52
Propionyl phosphate.....	0.65
Butyryl phosphate.....	0.76
Valeryl ".....	0.81
Caproyl ".....	0.86

* The *R_F* value is defined as the distance moved by the compound divided by the distance moved by the solvent. The values are reproducible within ± 0.05 unit. The developing solvent was water-saturated butanol; temperature 28°.

ture, 25 ml. of 95 per cent ethanol were added and the precipitated protein was removed by centrifugation. The supernatant was evaporated to a volume of 3 to 4 ml. on a steam bath and finally to dryness at room temperature. The residue was extracted with 2.0 ml. of absolute ethanol and the supernatant was concentrated to 0.1 to 0.2 ml. at room temperature. The sample was centrifuged and the supernatant was used for chromatographic analysis.

From 0.01 to 0.02 ml. of the sample was used on a 50 cm. strip of filter paper. The developing solvent was water-saturated butanol. The technique used in preparing and developing the paper chromatogram was the same as that employed by Hotchkiss (7).

The chromatogram was developed unidimensionally for 5 to 10 hours at 28°. After drying the paper at room temperature, the various hydroxamic acid spots were located by spraying the paper with the concentrated ferric chloride solution.

The hydroxamic acids were identified by their R_f values (Table I) and by comparison with synthetic hydroxamic acids.

To obtain a quantitative estimation of the various hydroxamic acids, the individual spots were cut from the paper strip and placed in colorimeter tubes. 6.0 ml. of the dilute ferric chloride solution were added and the solution was agitated. After 1 to 2 minutes, essentially all of the hydroxamic acid was extracted. The paper was removed and the optical density of the solution was measured with an Evelyn colorimeter equipped with a 540 $m\mu$ filter.

Sensitivity—Hydroxamic acids in amounts of 0.01 to 0.02 μM produce easily detectable spots when the above method is used. Satisfactory quantitative estimations can be made when the spots contain 0.1 to 3.0 μM .

TABLE II

Chromatographic Analysis of Mixtures of Acyl Phosphates with 2 to 6 Carbon Atoms

Compound	Acyl P added		Acyl P recovered								Average recovery
			Sample 1		Sample 2		Sample 3		Sample 4		
	μM	ratio	μM	ratio	μM	ratio	μM	ratio	μM	ratio	per cent
Acetyl P.....	17	1.00	15.3	1.00	15.0	1.00	15.6	1.00	14.4	1.00	89
Propionyl P.....	21	1.23	18.2	1.19	17.7	1.18			16.5	1.15	83
Butyryl P.....	22	1.29	19.5	1.27			21.7	1.39			94
Valeryl “.....	23	1.35			19.0	1.27					83
Caproyl “.....	21	1.23	19.2	1.25			17.2	1.10			87

Recovery—Presented in Table II are the results of experiments demonstrating the applicability of the method for estimating the relative amounts of acyl phosphates in mixtures. In these experiments, various combinations of synthetic acyl phosphates having 2 to 6 carbon atoms were analyzed by the above method. As shown in the last column of Table II, the average total recovery of the individual acyl phosphates after chromatographic separation was about 85 per cent.

Fortunately the loss was non-specific; good agreement (100 ± 5 per cent) was obtained between the ratios of added and recovered acyl phosphates. In practice, therefore, the total acyl phosphate concentration of the test solution was determined directly by the hydroxylamine method of Lipmann and Tuttle (17) and a separate aliquot of the test solution was analyzed by the chromatographic method to determine the relative proportions of the different compounds. From these data, it was possible to compute the actual concentrations of the individual acyl phosphates in the test solution.

Other methods used in the experimental work have already been described (15, 20-24).

Results

Transfer of Phosphoryl Group of Acetyl Phosphate to Adenylic Acid—To determine whether the phosphoryl group of acetyl phosphate could be transferred to adenylic acid, various mixtures of acetyl phosphate and adenylic acid were incubated with cell-free extracts. After various periods of incubation, the concentrations of acetyl phosphate and of pyrophosphate (7 minute-hydrolyzable phosphate) were determined. The

TABLE III

Transfer of Acetyl-Bound Phosphorus to Adenylic Acid

20 mg. of cell-free extract (Lot D) were incubated with the indicated amounts of acetyl phosphate and adenylic acid in an evacuated Thunberg tube at 26°. The total liquid volume was 2.0 ml., pH 7.0.

Acetyl P, initial	AMP, initial	Incubation time	Δ acetyl P	Δ acetyl P due to AMP	P ₇ *
μM	μM	min.	μM	μM	μM
0	0	80			0.8
19	0	80	3.6	0	1.2
19	10	80	8.9	5.3	10
96	0	80	13	0	0
96	0	155	23	0	0
96	10	80	30	17	17
96	10	155	37	14	16
96	20	155	51	28	38

* P₇ refers to the increase in inorganic phosphate obtained by 7 minutes hydrolysis in 0.1 N HCl at 100°.

results, presented in Table III, show that the disappearance of acetyl phosphate is increased by the addition of adenylic acid and that there is a corresponding increase in the amount of pyrophosphate. The data therefore indicate that the phosphoryl group of acetyl phosphate was transferred to adenylic acid (Reaction 5).



Comparable changes were not observed in a control sample (not shown in Table III) containing boiled enzyme. In those samples in which the initial acetyl phosphate concentration was 5 to 10 times greater than that of adenylic acid, the molar quantity of 7 minute-hydrolyzable phosphate formed was 1.5 or more times greater than that of adenylic acid added. These results indicate that transphosphorylation does not

stop at the ADP stage but that ATP is formed also. A direct confirmation of this was obtained from an experiment in which acetyl phosphate was incubated with ADP. The results (Table IV) show that when $5.8\ \mu\text{M}$ of ADP were incubated with $30\ \mu\text{M}$ of acetyl phosphate $3.4\ \mu\text{M}$ of 7 minute-hydrolyzable phosphate were formed and there was a corresponding increase in the amount of acetyl phosphate lost. It is evident therefore that the phosphoryl group of acetyl phosphate can be transferred to ADP (Reaction 6).



TABLE IV

Transfer of Acetyl-Bound Phosphorus to ADP

20 mg. of cell-free extract (Lot D) and the indicated substrates were incubated in a volume of 2.0 ml. for 130 minutes at 26° in an evacuated Thunberg tube.

Acetyl P, initial	ADP (P_7) added	P_7 , final	Acetyl P, final	Δ acetyl P due to ADP added	ΔP_7 due to ADP added
μM	μM	μM	μM	μM	μM
30	0	2.3	23	0	0
30	5.8	11.5	19	4	3.4

TABLE V

Transfer of Labile Phosphorus of ADP and ATP to Acetate

Experiment No.	Acetate, initial	ADP, initial	ATP, initial	P_7 , initial	Incubation time	Acetyl P, final	P_7 , final	ΔP_7
	μM	μM	μM	μM	min.	μM	μM	μM
1*	155	19.4	0	19.4	95	1.4	15.6	3.8
2†	155	0	0	0.8	75	0		
	155	0	8.2	16.4	75	1.2	12.5	3.9

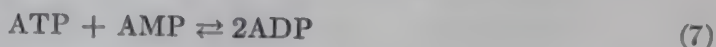
* 50 mg. of Lot D, cell-free extract, and the indicated substrates in 2.0 ml. volume were incubated in an evacuated Thunberg tube, 26° .

† 25 mg. of Lot D and the indicated substrates in 1.5 ml. volume were incubated in an evacuated Thunberg tube, 26° .

Transfer of Labile Phosphorus of Adenyl Pyrophosphates to Acetate—To determine whether the transphosphorylations described by Reactions 5 and 6 are reversible, mixtures of ADP and acetate and ATP and acetate were incubated with the enzyme preparation. The results (Table V) show that small but significant amounts of acetyl phosphate were formed. The amounts produced were of the same order of magnitude as those observed by Lipmann (13) in his studies with enzyme preparations of *C. butylicum* and *L. delbrueckii*. From these results it may be concluded that Reactions 5 and 6 are reversible.

It is probable that in these studies a final equilibrium was not attained from either side owing to complicating side reactions. It is obvious, however, that the equilibrium favors the formation of adenylyl pyrophosphates.

Data similar to those obtained in the above experiments were used by Lipmann (10, 13) to calculate the equilibrium constant for the reaction. For these calculations, it was assumed that ADP, not adenylic acid, was the true phosphoryl group acceptor (Reaction 3). This assumption was based upon the observation by Meyerhof *et al.* (18) (see also Bücher (4)) that in a similar reaction between 1,3-diphosphoglyceric acid and the adenylic acid system ADP and not adenylic acid was the true acceptor. Accordingly, Lipmann concluded that when adenylic acid is active, as it was in his enzyme preparations, probably a phosphodismutation, with some ATP present in the preparation, yields ADP in the manner described by Kalckar (9) (Reaction 7), and that the ADP thus formed is the true acceptor of the phosphoryl group of acetyl phosphate.

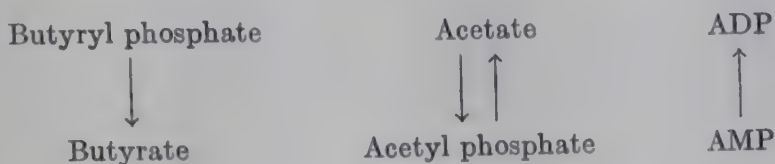


On the basis of these assumptions, Lipmann obtained an equilibrium constant, $K = (\text{ATP} \times \text{acetate})/(\text{ADP} \times \text{acetyl P}) = 177$, for Reaction 6. A similar calculation (see Lipmann (13) for details) from the data of Experiment 2, Table V, gave a value of 360. Considering the many assumptions made in these calculations and the probable failure to achieve a true equilibrium, the agreement between the latter value and that obtained by Lipmann is not unreasonable.

Myokinase Activity—Since the method of calculating the equilibrium constant for the reaction between acetyl phosphate and the adenylic acid system assumes a rapid phosphodismutation of adenosine diphosphate, it seemed desirable to determine experimentally whether the latter reaction actually occurs in the enzyme preparations of *C. kluyveri*. This was done by incubating a mixture of AMP and ATP with the enzyme preparation and looking for a disappearance of AMP (Reaction 7). AMP was estimated by the method of Kalckar (9, 26) and the adenylyl compounds in the barium-soluble and insoluble fractions were estimated by a pentose determination after acid hydrolysis (26).

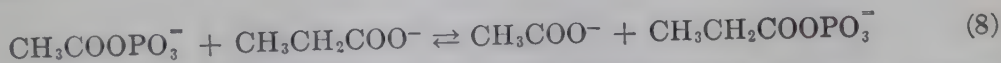
The results were entirely negative. It is therefore probable that in the transphosphorylation between acetyl phosphate and the adenylic acid system both adenylic acid and adenosine diphosphate can function directly as phosphoryl group acceptors. In view of these results, it is obvious that calculations of the type used by Lipmann to determine the equilibrium constant of the transphosphorylation between acetyl phosphate and ADP are inadequate when applied to the *C. kluyveri* system.

In other experiments it has been shown that the enzyme preparations catalyze the transfer of the phosphoryl group of butyryl phosphate to adenylic acid. However, in view of the fact that a direct transphosphorylation probably occurs between butyryl phosphate and acetate (see below) one cannot conclude that transfer of the phosphoryl group from butyryl phosphate to adenylic acid is direct. It is possible that the small amount of acetate present in the enzyme preparation serves as an intermediate phosphoryl group carrier, as indicated in the following scheme.



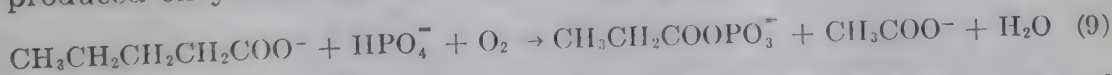
Evidence that such a mechanism may contribute to the transfer of the phosphoryl group from butyryl phosphate to adenylic acid was obtained by showing that the addition of acetate to a mixture of butyryl phosphate and adenylic acid increased the rate of transphosphorylation about 20 per cent. A final decision as to the mechanism of this reaction must await the preparation of a more highly purified enzyme that does not contain catalytic amounts of acetate.

Reversible Transfer of Phosphoryl Group of Acetyl Phosphate to Propionate—Summarized in Table VI are the results of several experiments showing that enzyme preparations of *C. kluyveri* catalyze a rapid reversible transphosphorylation between acetyl phosphate and propionate (Reaction 8).



In Experiments 1 and 2, the enzyme was incubated with acetyl phosphate and propionate. After various periods of incubation, the test solution was analyzed chromatographically for acetyl phosphate and propionyl phosphate. The results show that there was a rapid decrease in the acetyl phosphate concentration and an increase in the concentration of propionyl phosphate. In Experiment 3, propionyl phosphate was incubated with acetate and a rapid transfer of the phosphoryl group to acetate was observed.

In Experiment 4 (Table VI), acyl phosphates were not added but were produced enzymatically by the oxidation of valeric acid (Reaction 9).



The steam-volatile acids produced in this oxidation are propionic and acetic. 1 mole of acyl phosphate is also formed. It is not known at present whether the immediate products of the reaction are propionyl

phosphate and acetate, as indicated in Reaction 9, or acetyl phosphate and propionate. In any event the data of Experiment 4, Table VI, show that a rapid transphosphorylation occurs, forming ultimately both propionyl and acetyl phosphates in almost equal amounts.

These experiments demonstrate very clearly the reversible nature of the transphosphorylation on the acyl phosphate level. The attainment of a true equilibrium is indicated by the fact that essentially the same

TABLE VI

Reversible Transfer of Phosphoryl Group of Acetyl Phosphate to Propionate

Experiments 1 and 2, 125 mg. of dried cells (Lot I), pH 7.5, suspended in 5 ml. of water, were incubated anaerobically with the indicated substrates at 26°.

Experiment 3, 75 mg. of cell-free extract (Lot E), pH 7.5, dissolved in 5 ml. of water, were incubated anaerobically with the indicated substrates at 26°.

Experiment 4, 50 mg. of dried cells (Lot I) and 50 μ M of sodium valerate in 2 ml. of 0.1 M phosphate buffer, pH 8.0, were shaken with air in a Warburg vessel for 75 minutes at 26°. During incubation, 41.6 μ M of oxygen were consumed and 38.9 μ M of acyl phosphate were formed.

Experiment No.	Incubation time	Acetate	Propionate	Propionyl P	Acetyl P	$K = \frac{\text{propyl P} \times \text{acetate}}{\text{acetyl P} \times \text{propionate}}$
	<i>min.</i>	μ M per ml.	μ M per ml.	μ M per ml.	μ M per ml.	
1	0	1.7	20	0	30	
	15	14.1	8.6	11.4	17.6	1.06
2	0	3.5	40	0	40	
	5	22.2	21.3	18.7	21.3	0.92
	15	23.3	21.2	18.8	20.2	1.10
	180	30.6	27.6	12.4	12.9	1.06
3	0	14.7	4.5	16.0	0	
	5	8.9	10.5	10.0	5.8	1.29
	30	7.7	10.7	9.8	6.0	1.17
	60	7.5	10.9	9.6	6.2	1.07
4	0	3.5	0	0	0	
	75	25.3	22.5	19.1	19.8	1.08

equilibrium constant for Reaction 8, $K = (\text{propionyl P} \times \text{acetate})/(\text{acetyl P} \times \text{propionate})$, was obtained in all of the above experiments (see the last column of Table VI). The value of the equilibrium constant indicates that the phosphate bond energies in propionyl and acetyl phosphates are nearly identical. The rate of the transphosphorylation reaction is evidently very high, since almost complete equilibration of the two acyl phosphates was reached within 5 minutes.

Reversible Transfer of Phosphoryl Group of Acetyl Phosphate to Butyrate—Koepsell *et al.* (12) have reported a rapid and extensive trans-

phosphorylation between acetyl phosphate and butyrate by cell-free extracts of *C. butylicum*. It seemed probable, therefore, that a similar transphosphorylation would be catalyzed by extracts of *C. kluyveri*, especially since a rapid reaction occurs between acetyl phosphate and propionate. The data of Table VII show that such a phosphate trans-

TABLE VII

Reversible Transfer of Phosphoryl Group of Acetyl Phosphate to Butyrate

Experiments 1 and 2, 125 mg. of dried cells of *C. kluyveri* (Lot I), pH 7.6, suspended in 5 ml. of water, were incubated anaerobically with the indicated substrates at 26°.

Experiments 3 and 4, 90 mg. of cell-free extract of *C. butylicum* (Koepsell *et al.* (12)), pH 6.8, were dissolved in 3.6 ml. of water and incubated anaerobically with the indicated substrates at 37°.

Experiment No.	Incubation time	Acetate	Butyrate	Acetyl P	Butyryl P	$K = \frac{\text{butyryl P} \times \text{acetate}}{\text{acetyl P} \times \text{butyrate}}$
	min.	$\mu\text{M per ml.}$	$\mu\text{M per ml.}$	$\mu\text{M per ml.}$	$\mu\text{M per ml.}$	
1	0	3.5	40	40	0	
	15	6.3	39.2	37.2	0.8	0.003
	60	9.6	38.9	33.9	1.1	0.008
	120	14.5	39.0	29.0	1.0	0.013
	180	17.9	39.1	25.6	0.9	0.016
2	0	43.5	2.0	0	38.0	
	35	39.4	20.5	4.1	19.5	9.2
	90	40.4	30.1	3.1	9.9	4.3
	195	40.4	37.9	3.1	2.1	0.73
3	0	12.9	58.5	35.6	0	
	30	31.1	54.1	27.4	4.0	0.08
	90	37.7	53.0	20.8	5.2	0.18
	195	44.0	53.0	14.5	5.2	0.30
	315	49.6	51.4	8.9	4.1	0.42
4	0	45.0	27.0	0	31.1	
	45	44.1	30.5	0.9	27.5	44
	115	43.4	33.5	1.7	24.5	18.6
	215	42.8	36.9	2.2	21.1	11.2

fer does occur; however, the rate of the reaction was very slow and in no instance was it possible to observe a considerable net formation of butyryl phosphate from acetyl phosphate and butyrate or of acetyl phosphate from butyryl phosphate and acetate. Thus, in Experiment 1, Table VII, starting with 40 μM of acetyl phosphate and 40 μM of butyrate, about 1 μM of butyryl phosphate was produced after 15 minutes incubation, but no further increase was obtained even after 3 hours, although the acetyl phosphate was decreased by phosphatase action. These re-

sults could be interpreted to mean that the enzyme catalyzing the transphosphorylation was inactivated after 15 minutes. However, this is probably not true, since the constancy of the butyryl phosphate concentration in a preparation containing an active butyryl phosphatase (Table VII) indicates that transphosphorylation was taking place throughout the experiment. The possibility that acetyl phosphate in relatively high concentration inhibits the hydrolysis of butyryl phosphate by phosphatase action was investigated and no such effect could be observed.

In Experiment 2, Table VII, the reverse reaction, the transfer of the phosphoryl group from butyryl phosphate to acetate, was studied. Again a relatively slow transphosphorylation was observed. After 35 minutes, $4.1\ \mu\text{M}$ of acetyl phosphate had been produced but longer incubation did not cause a further accumulation of acetyl phosphate, although the butyryl phosphate level declined rapidly and only $2.1\ \mu\text{M}$ were left after 195 minutes, at which time there were still $3.1\ \mu\text{M}$ of acetyl phosphate.

The above results show that a true equilibrium was not attained in the acetyl phosphate-butyrate reaction, probably because of the decomposition of the acyl phosphates by phosphatase action. Not only is the rate of hydrolytic cleavage of the acyl phosphates of the same order of magnitude as the rate of transphosphorylation, but in addition the two acyl phosphates are decomposed at markedly different rates. It will be shown below that the hydrolysis of butyryl phosphate is 3.5 times more rapid than the hydrolysis of acetyl phosphate. It should be emphasized that these considerations do not apply to the acetyl phosphate-propionate transphosphorylation, since the rate of transphosphorylation between these substrates is very much greater than the rate of phosphatase action. Moreover, the rates of enzymatic hydrolysis of acetyl phosphate and propionyl phosphate are more nearly the same. The observed equilibrium constant for the acetyl phosphate-propionate reaction is undoubtedly fairly reliable.

Similar studies have shown that a transphosphorylation also occurs between acetyl phosphate and valeric and caproic acids. The rate of transphosphorylation to these acids is somewhat slower than to butyrate.

In studies of the transphosphorylation between acetyl phosphate and butyrate with enzyme preparations of *C. butylicum* Koepsell *et al.* (12) found as much as 30 per cent of the total acyl phosphate as butyryl phosphate. This suggested that the true equilibrium for the reaction might be much more in favor of butyryl phosphate than our results with *C. kluyveri* enzymes indicated. The higher percentage of butyryl phosphate observed with *C. butylicum* could be due to a more favorable ratio between the rates of transphosphorylation and dephosphorylation.

Fortunately we were able to obtain a small sample of the *C. butylicum* preparation originally used by Koepsell and Johnson² (11) and to compare its behavior directly with that of the *C. kluyveri* preparations (Experiments 3 and 4, Table VII). The results demonstrate that the *C. butylicum* preparation, though 7 years old, was still able to catalyze a transphosphorylation between acetyl phosphate and butyrate. The data of Experiment 3 show that after 90 minutes incubation of the enzyme with a mixture of acetyl phosphate and butyrate 20 per cent of the total acyl phosphate was the butyrate derivative. This is a much larger percentage of butyryl phosphate than was obtained with *C. kluyveri* preparations, and also the rate of transphosphorylation was significantly greater. However, the rate was not enough greater than the rate of the interfering hydrolytic reactions to permit a reliable determination of the equilibrium constant. This constant cannot be established with certainty until the transphosphorylating enzyme preparation has been freed of phosphatase activity.

Rôle of Adenylic Acid in Synthesis and Oxidation of Butyrate—It has been pointed out already that a postulated mechanism for caproic acid synthesis involves a series of transphosphorylation reactions, as indicated by Reactions 2 and 3. In the preceding experiments it was shown that reactions of this type are catalyzed by enzyme preparations of *C. kluyveri*. It appears possible therefore that these reactions actually are involved in fatty acid synthesis as postulated. In the course of these studies, however, it was found that not all enzyme preparations were equally able to catalyze the transphosphorylations. For example, with the cell-free enzymes of Lots C and D' (20), the transfer of the phosphoryl group of acetyl phosphate to adenylic acid was very rapid, being almost complete after 10 minutes of incubation. The corresponding transphosphorylation with the dried cell preparation of Lot H was relatively slow and the reaction did not occur at all with the dried cells or cell-free enzymes of Lots G, E, or E'. The possibility that the latter preparations contained an inhibitor was excluded, since the addition of these preparations to the cell-free extracts of Lot D produced no change in the rate of transphosphorylation by the latter. Although the enzyme preparations of Lot G were unable to catalyze a transfer of the phosphoryl group of acetyl phosphate to adenylic acid or adenosine diphosphate, they were able to catalyze the synthesis and oxidation of caproate. It must be concluded that transphosphorylations involving the adenylic acid system do not participate in caproate synthesis or oxidation.

The possibility that adenylic acid served as an intermediate carrier

² We are indebted for this preparation to Dr. E. J. Ordal of the University of Washington, who obtained it from Dr. Koepsell.

in the transfer of the phosphoryl group of acetyl phosphate to butyrate (Reactions 2 and 3) was excluded by the observation that the cell-free extract of Lot G could catalyze a transphosphorylation between acetyl phosphate and butyrate but could not catalyze a transfer to adenylic acid or adenosine diphosphate. These results suggest that the phosphoryl group can be transferred directly from acetyl phosphate to other fatty acids. In any event adenylic acid and adenosine diphosphate are not obligatory phosphate carriers.

Rôle of Butyryl Phosphate in Synthesis and Oxidation of Caproate—While the above experiments appear to rule out adenylic acid and its derivatives as important components of the system causing the synthesis and oxidation of fatty acids, it still appeared possible that butyryl phosphate was involved in these reactions.

If butyryl phosphate is a normal intermediate in the oxidation of caproate (the reverse of Reaction 4), one would expect it to accumulate to some extent when caproate is oxidized aerobically. Therefore, an experiment was done in which the acyl phosphates produced in the oxidation of caproate were examined for the presence of butyryl phosphate. 20 μM amounts of caproate were incubated with the enzyme preparation (dried cells, Lot I) in Warburg vessels in air. When one-half (20 μM) and three-quarters (30 μM) of the theoretical oxygen had been consumed, 14 and 26 μM of acyl phosphate were formed, respectively, and the oxidation was stopped by the addition of hydroxylamine reagent. The enzyme mixtures were analyzed chromatographically for acetyl and butyryl phosphates. In neither sample was it possible to detect even trace amounts of butyryl phosphate. Practically all of the acyl phosphate present was acetyl phosphate. A trace amount of caproyl phosphate, probably formed by secondary transphosphorylation, was also detected. The absence of butyryl phosphate indicates, but does not definitely prove, that this compound is not a normal product of caproate oxidation. The possibility remains that butyryl phosphate was preferentially oxidized further to acetyl phosphate and acetate and therefore failed to accumulate. To explore this possibility, experiments were done to compare the rates of oxidation of caproate and synthetic butyryl phosphate. It was found that the two compounds are oxidized at about the same rate, and therefore it is unlikely that a preferential utilization of butyryl phosphate can account for the failure to detect this compound as a product of caproate oxidation.

Reduction of Acyl Phosphates to Their Corresponding Alcohols—To determine whether butyryl phosphate could be an intermediate in the synthesis of caproate (Reaction 4) an experiment was performed in which butyryl phosphate and acetate were incubated with the enzyme

preparation in an atmosphere of hydrogen. The results showed that hydrogen was taken up. However, almost as much hydrogen was consumed by a sample containing butyryl phosphate alone ($36.4 \mu\text{M}$) as by a mixture of butyryl phosphate and acetate ($49 \mu\text{M}$). Since relatively small amounts of acetate (2 to $4 \mu\text{M}$) were present in the enzyme preparation, it is obvious that the reduction of butyryl phosphate alone could not be due to the formation of caproate. It appeared probable, therefore, that the butyryl phosphate had been reduced to butanol. Since propanol is found as a normal product of the fermentation of ethanol and propionate by growing cultures of *C. kluyveri* (3, 25), it also seemed likely that this substance was formed by reduction of propionyl phosphate.

TABLE VIII

Reduction of Acyl Phosphates to Corresponding Alcohols

Each Warburg vessel contained 50 mg. of dried cells (Lot I), 0.1 M trishydroxymethylaminomethane buffer (pH 7.6), and $100 \mu\text{M}$ of the acyl phosphates. Total liquid volume, 2.0 ml.; gas phase, H_2 .

Experiment No.	Acyl P	Rate of H_2 uptake, initial, per 15 min.	H_2 uptake, total	Alcohol	
				Theoretical	Found*
		<i>c.mm.</i>	μM	μM	μM
1	Propionyl P	132	111	55.5	38 (Propanol)
	Butyryl P	116	33		
	Caproyl "	77	32		
2†	Butyryl "		151	75.5	58 (Butanol)

* Estimated by the method of Johnson (8).

† In Experiment 2, the contents of eight similar Warburg vessels were combined in order to provide sufficient material to analyze.

Proof that the acyl phosphates are reduced to the corresponding alcohols was obtained in other experiments. The data of Table VIII show that propionyl, butyryl, and caproyl phosphates are reduced with molecular hydrogen. The rate of hydrogen uptake varies inversely with the length of the carbon chain. Thus the relative rates of reduction of propionyl, butyryl, and caproyl phosphates were 100, 88, and 58, respectively. After hydrogen uptake had ceased, the propionyl and butyryl phosphate samples were adjusted to pH 9.0 and distilled. The volatile alcohols recovered in the distillates were oxidized to their corresponding fatty acids with acid dichromate (8). Duclaux distillation of the fatty acids showed that almost pure propionic and butyric acids were derived from the propionyl and butyryl phosphate samples, respectively. Small amounts of acetate (about 5 per cent) also found were undoubtedly

formed, mainly by overoxidation of the alcohols with the acid dichromate (27). The total amount of acid recovered from the alcohol oxidations was approximately 75 per cent of that to be expected if all of the hydrogen consumed was utilized in the reduction of the acyl phosphates to the corresponding alcohols (Reaction 10).



The fact that butyryl phosphate is more readily converted to butyl alcohol than to caproic acid is a strong argument against the idea that butyryl phosphate is a normal intermediate in the metabolism of *C. kluyveri*, because butyl alcohol is never formed as a normal fermentation product.

Hydrolytic Decomposition of Acyl Phosphates by Phosphatase Action—Throughout this investigation and in previous studies with enzymes of *C. kluyveri* (20–24) reference has been made to the fact that the enzyme preparations contain active acyl phosphatases and the failure to observe a perfect stoichiometric relationship in reactions involving acetyl or other acyl phosphates was due to the enzymatic hydrolysis of these compounds. It seemed desirable, therefore, to present the results of experiments designed to demonstrate the magnitude of this effect.

Since the hydrolysis of an acyl phosphate leads to the formation of 1 equivalent of acid (Reaction 11), the reaction can be followed manometrically if it is carried out in a bicarbonate buffer.



The results of an experiment on the rates of hydrolysis of the acyl phosphates of fatty acids containing from 2 to 6 carbon atoms are given in Table IX. Experiment 1 shows that the rate of hydrolysis increases with the number of carbon atoms, reaching a maximum with valeryl phosphate which is decomposed about 4 times more rapidly than acetyl phosphate. Phosphatase activity is completely destroyed by heating the enzyme preparation at 100° for 10 minutes. In this respect, the enzyme differs from the relatively thermostable animal acylphosphatase obtained from horse muscle (14).

Of some significance was the observation that the relative rates of hydrolysis of different acyl phosphates were not the same for different enzyme preparations. For example, in Experiment 1, Table IX, in which dried cells of Lot I were used, the enzymatic hydrolysis of butyryl phosphate was about 3.5 times more rapid than that of acetyl phosphate. However, with the cell-free extract of Lot E (Experiment 2), the rate was roughly 10 times as great. These results suggest that acetyl phosphate and butyryl phosphate are decomposed by different enzymes.

In order to obtain further information on this possibility the rates of hydrolysis of mixtures of acyl phosphates were compared with the rates of hydrolysis of the individual compounds. The results of two such experiments are given in Table X. In Experiment 1, the rates of hydrolysis of acetyl phosphate, valeryl phosphate, and a mixture of the two were compared. The decomposition of the acyl phosphate was measured manometrically by carbon dioxide evolution from a bicarbonate

TABLE IX

Enzymatic Hydrolysis of Acyl Phosphates

Each Warburg vessel contained 0.22 ml. of 0.1 M NaHCO_3 , 20 μM of acyl phosphate, and 50 mg. of dried cells (Lot I) or 30 mg. of cell-free extract (Lot E) where indicated. The total liquid volume was 2.0 ml. The gas phase was nitrogen containing 5 per cent CO_2 ; pH 8.0; temperature, 26°.

Experiment No.	Compound	Enzyme lot	Rate of CO_2 evolution, observed, per 15 min.*	Rate of CO_2 evolution due to phosphatase action, per 15 min.†
			<i>c.mm.</i>	<i>c.mm.</i>
1	Acetyl P	I (Boiled)	2.4	
	" "	"	26	13
	Propionyl P	"	40	27
	Butyryl P	"	58	45
	Valeryl "	"	64	51
	Caproyl "	"	46	33
	" " + valeryl P	" (Boiled)	5.1	
	None	" "	0	
2	"	"	11	
	Acetyl P	E	11	8
	Butyryl P	"	87	79
	" "	" (Boiled)	4.6	

* Based on the second 15 minute period of incubation.

† Data corrected for spontaneous hydrolysis of the acyl phosphate (about 3 c.mm. per 15 minutes) and for the enzyme blank.

solution and also by direct colorimetric measurement (17). The data show that the rate of decomposition of the mixture was considerably greater than the rate with either substrate alone, and in fact was almost equal to the sum of the two individual rates. This shows that acetyl phosphate and valeryl phosphate are non-competitive substrates and indicates that they are decomposed by different enzymes. In Experiment 2, the rates of hydrolysis of valeryl phosphate, caproyl phosphate, and a mixture of the two were compared. With these compounds the rate of hydrolysis of the mixture was intermediate between the individual rates, thus indicating a competition of the substrates for a single

enzyme. Considering all the available information, it may be concluded that at least two distinct phosphatases, differing in their ability to hydrolyze acetyl phosphate and the higher acyl phosphates, are active in the enzyme preparations. The simplest interpretation would be that one enzyme is specific for acetyl phosphate, whereas the other can attack all of the acyl phosphates or is specific for only the higher homologues.

Arsenolytic Decomposition of Acetyl Phosphate

Effect of Arsenate on Hydrolysis of Acetyl Phosphate—Mention has already been made of the fact that arsenate is a powerful inhibitor of cer-

TABLE X

Specificity of Acylphosphatase Activity

Each Warburg vessel contained 0.22 ml. of 0.1 M NaHCO_3 , 50 mg. of dried cells (Lot I), and the indicated amounts of the acyl phosphate. The total liquid volume was 2.0 ml. Gas phase, 5 per cent CO_2 in nitrogen; temperature 26° .

Experiment No.	Acetyl P, initial	Valeryl P, initial	Caproyl P, initial	Rate of CO_2 evolution per 15 min.	Total CO_2 evolved per 105 min.	Δ acyl P per 105 min.
	μM	μM	μM	c.mm.	μM	μM
1	0	0		*	1.4	
	17	0		19	7.6	6.1
	34	0		18	6.5	6.7
	0	20		49	16.2	15.6
	0	40		47	16.4	15.7
	17	20		63	19.1	21.0
2		0	0	*	2.1	
		0	17	38	14.6	12.5
		0	34	35	14.5	11.2
		20	0	49	17.2	16.9
		20	17	39	14.5	14.6

* Data corrected for CO_2 evolution in the control sample.

tain reactions catalyzed by *C. kluyveri*. For example, arsenate prevents the phosphoroclastic splitting of acetoacetate, the oxidation of butyrate and vinyl acetate, and the reduction of acetyl phosphate to butyrate. However, it has no effect on the reduction of acetoacetate to β -hydroxybutyrate. The latter fact indicates that arsenate does not influence the hydrogen-activating system and its effect is probably more intimately concerned with the reactions of acetyl phosphate.

In addition to the reduction of acetyl phosphate to butyrate, two other reactions involving acetyl phosphate have been demonstrated; namely, the transfer of the phosphoryl group to adenylic acid and adenosine diphosphate and the hydrolysis of acetyl phosphate to acetate and inorganic

phosphate. Therefore, experiments were done to ascertain whether arsenate has any effect on either of these reactions. Somewhat unexpected was the discovery that arsenate catalyzed the complete and very rapid decomposition of acetyl phosphate. Thus, when $17\text{ }\mu\text{M}$ of acetyl phosphate were incubated with the enzyme preparation in the presence of 0.0025 M arsenate, no acetyl phosphate could be detected after 15 minutes. With 0.02 M arsenate, none was left after 5 minutes. This catalytic effect of arsenate on acetyl phosphate decomposition was completely absent when boiled enzyme preparations were used.

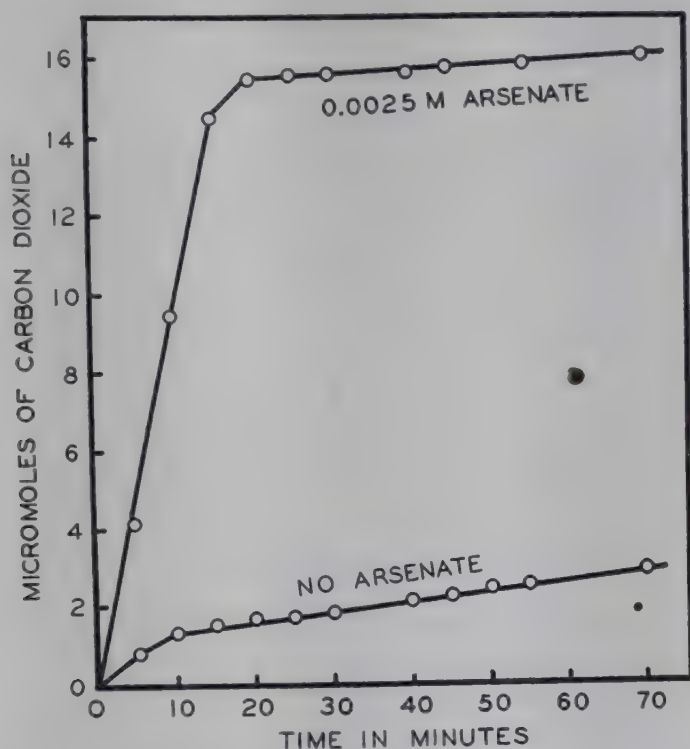


FIG. 1. Effect of arsenate on enzymatic hydrolysis of acetyl phosphate. Each Warburg vessel contained $16\text{ }\mu\text{M}$ of acetyl phosphate, 30 mg. of cell-free extract (Lot E), and arsenate as indicated in 2.0 ml. of 0.11 M NaHCO_3 . The gas phase was 6 per cent CO_2 in N_2 ; temperature, 26° .

If the disappearance of acetyl phosphate catalyzed by arsenate is due to hydrolysis, 1 mole of acid would be formed (Reaction 11) and the reaction could be followed manometrically in a bicarbonate solution. The data of an experiment carried out to investigate this possibility are plotted in Fig. 1 which shows that the decomposition of acetyl phosphate in the presence of arsenate results in the liberation of 1 equivalent of acid, in agreement with Reaction 11.

Specificity of Arsenolysis of Acyl Phosphates—In view of the large catalytic effect of arsenate on the hydrolysis of acetyl phosphate, it

seemed desirable to determine whether arsenate has a similar effect on the hydrolysis of the higher acyl phosphates. The experimental results presented in Table XI show that a very powerful catalytic effect of arsenate (26- to 65-fold) was attained only with acetyl phosphate. With the higher homologues a much smaller stimulation of the hydrolysis rate (1.1- to 2.5-fold) was observed.

The hydrolysis of propionyl phosphate in the presence of arsenate was increased only slightly by the addition of acetate (Experiment 2, Table XI). Since this enzyme preparation catalyzes a rapid transphosphorylation between propionyl phosphate and acetate (Table VI), one would expect that the acetyl phosphate produced by transphosphory-

TABLE XI

Arsenolytic Decomposition of Various Acyl Phosphates

Each Warburg vessel contained 0.11 M NaHCO_3 and 20 μM of acyl phosphate. The total liquid volume was 1.0 ml. Gas phase, 5 per cent CO_2 in nitrogen; temperature, 26°.

Experiment No.	Acyl P	Rate of CO_2 evolution	
		No arsenate	0.0025 M arsenate
		c.mm. per 5 min.	c.mm. per 5 min.
1. 50 mg. dried cells (Lot I)	Acetyl P	8.5	225
	Butyryl P	19	41
	Valeryl "	21	31
	Caproyl "	15	30
2. 30 mg. cell-free ex ₂ tract (Lot E)	Acetyl P	2.6	168
	Propionyl P	8	21
	Butyryl P	29	32
	Propionyl P + ace- tate, 30 μM	9	43

lation would be decomposed rapidly by arsenolysis. The observed slow hydrolysis therefore suggested that propionyl phosphate or propionate, or both, inhibits the arsenolysis of acetyl phosphate. An experiment was performed to test directly the effect of these and other substances on the arsenolysis of acetyl phosphate. The results given in Table XII show clearly that propionyl phosphate is a strong specific inhibitor of the arsenolysis of acetyl phosphate. The other substances tested, namely acetate, propionate, butyrate, and butyryl phosphate, had only slight effects. Propionate is a special case, however. If this substance is added to the enzyme at the same time as arsenate, it has little effect on the arsenolysis of acetyl phosphate (Sample 3, Table XII). If, however, the propionate is allowed to incubate with the enzyme for some time prior to addition of the arsenate (Sample 7), a marked inhibition of

acetyl phosphate decomposition is observed, owing undoubtedly to the fact that considerable propionyl phosphate is formed by transphosphorylation.

In other experiments that will not be described in detail, it was shown that the inhibition of propionyl phosphate cannot be overcome by the addition of more acetyl phosphate. The inhibition depends upon the relative concentration of propionyl phosphate and arsenate. Inhibition occurs when the molar ratio of propionyl phosphate to arsenate is 2:1 or above; when the ratio is 1:1.5, there is almost no inhibition. The

TABLE XII

Specific Inhibition of Arsenolytic Decomposition of Acetyl Phosphate by Propionyl Phosphate

Each Warburg vessel contained 15 mg.* of cell-free extract (Lot E), 0.11 M NaHCO_3 , 0.0025 M arsenate, and 16 μM of acetyl phosphate. The total liquid volume was 2.0 ml. Gas phase, 95 per cent H_2 -5 per cent CO_2 ; temperature, 26°.

Sample No.	Inhibitor	Rate of CO_2 evolution per 15 min.	
		<i>c.mm.</i>	
1	None	131	
2	Acetate, 40 μM	106	
3	Propionate, 40 μM	125	
4	Butyrate, 40 μM	103	
5	Butyryl P, 18 μM	113	
6	Propionyl P, 16 μM	38	
7†	Propionate, 40 μM	51	

* In this experiment only 15 mg. of enzyme were used in order to slow down the arsenolysis reaction to a point at which accurate rate measurements could be made.

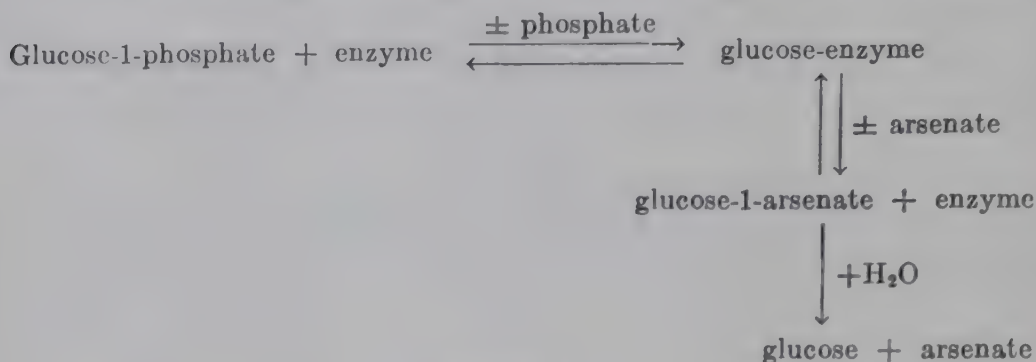
† The propionate and the acetyl phosphate were both incubated with the enzyme during a 25 minute equilibration period prior to dumping the arsenate from the side arm. In all other samples, the acetyl phosphate was incubated with the enzyme during the equilibration period and the inhibitor and arsenate were both placed in the side arm and mixed with the enzyme simultaneously after equilibration.

results indicate that propionyl phosphate inhibition of the arsenolysis of acetyl phosphate is competitive with respect to arsenate rather than acetyl phosphate.

At low enzyme concentrations (0.75 per cent) at which the arsenolysis of acetyl phosphate is fairly slow and can be measured accurately, it has been observed repeatedly that the rate of arsenolysis changes abruptly after about 50 to 75 per cent of the acetyl phosphate has been decomposed. This is not a gradual change. During the first period of decomposition (usually 20 to 40 minutes) the rate is constant, but then it changes very rapidly, within about 5 minutes, to another rate that is

twice as great. So far no adequate explanation of this effect has been developed. In view of this effect, it should be mentioned that all decomposition rates given in this paper refer to the initial rates.

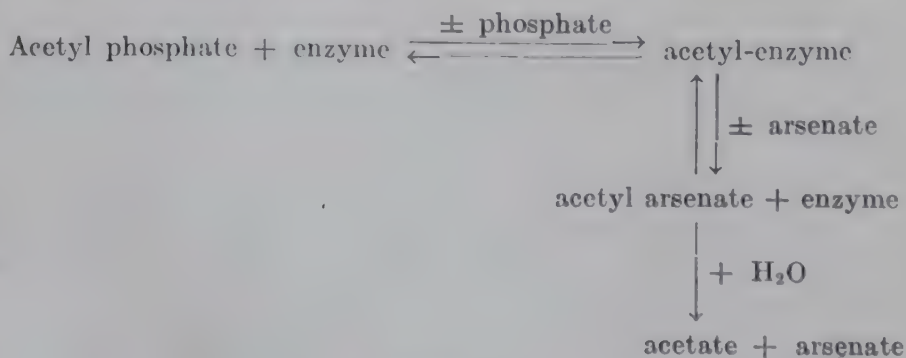
The catalytic effect of arsenate on the hydrolysis of acetyl phosphate is strongly reminiscent of a similar effect of arsenate on the hydrolysis of glucose-1-phosphate by the glucose-transferring enzyme obtained from the bacterium *Pseudomonas saccharophila* (6). In the latter instance, the course of the arsenolytic decomposition is represented in Scheme I.



SCHEME I

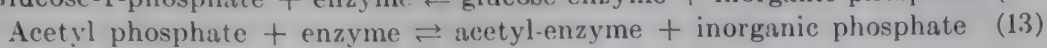
According to Scheme I the enzyme is in reality a *trans*-glucosidase whose function is to accept glucose from a suitable donor such as glucose-1-phosphate and transfer it to an appropriate acceptor which may be any one of several monosaccharides, inorganic phosphate, or arsenate. When arsenate is the acceptor, it is believed that glucose-1-arsenate is formed and that this substance undergoes a rapid spontaneous hydrolysis to glucose and arsenate. Thus, the net effort is a hydrolysis of glucose-1-phosphate to glucose and inorganic phosphate.

The arsenolytic decomposition of acetyl phosphate by the cell-free extracts of *C. kluyveri* can be represented in an analogous way (Scheme II) if it is assumed that the reaction is catalyzed by an acetyl transferring enzyme or *trans*-acetylase.



SCHEME II

Exchange of Acetyl-Bound and Inorganic Phosphate—According to Schemes I and II the primary reactions are reversible (Reaction 12 and 13.)



Doudoroff, Barker, and Hassid (5) obtained evidence in support of Reaction 12 by incubating glucose-1-phosphate with the enzyme preparation of *P. saccharophila* in the presence of P^{32} -labeled inorganic phosphate and observed a rapid exchange of phosphate between the ester and the inorganic form.

TABLE XIII

Exchange of Inorganic and Acetyl-Bound Phosphorus

Each Thunberg tube contained 25 mg. of cell-free extract (Lot G) and 50 μM each of acetyl phosphate and P^{32} -labeled inorganic phosphate (specific activity about 30,000 c.p.m. per μM) in a total liquid volume of 3.0 ml. The enzyme used in the control sample had been inactivated by heating at 100° for 10 minutes. pH 8.0. After incubating at 26° for 15 minutes in the evacuated Thunberg tubes, the inorganic phosphate was separated from the acetyl phosphate by calcium precipitation; phosphate determinations (Fiske-Subbarow) and the radioactivity measurements were made on suitable aliquots of the calcium-insoluble fraction (redissolved in dilute HCl) and on the calcium-soluble fraction.

Enzyme	Quantity recovered after incubation	Specific activity after incubation
	μM	c.p.m. per μM
Boiled (control). Inorganic P.....	63	30,500
“ “ Acetyl P.....	45	100
Active. Inorganic P.....	61	17,400
“ Acetyl P.....	42	15,700

A similar experiment was performed with the cell-free extract of *C. kluyveri* to determine whether Reaction 13 is tenable. Acetyl phosphate was incubated with the enzyme in the presence of P^{32} -labeled inorganic phosphate. After incubation, the acetyl phosphate and inorganic phosphate were separated and analyzed for radioactivity. A similar sample containing an inactive enzyme preparation, obtained by heating at 100° for 10 minutes, was incubated as a control. The data (Table XIII) show that after 15 minutes incubation with the active enzyme the molar specific activity of the acetyl-bound phosphate was 90 per cent of that of the inorganic phosphate. Evidently an almost complete equilibration of the phosphate in the two forms had been achieved. Relatively little radioactivity was observed in the acetyl phosphate isolated from

the control sample, indicating that the phosphate exchange was enzyme-catalyzed. The small amount of P^{32} in the acetyl phosphate fraction of the control sample was probably due to a slight contamination with inorganic phosphate.

A perfect parallelism thus exists between the reactions of acetyl phosphate catalyzed by the extracts of *C. kluyveri* and those of glucose-1-phosphate catalyzed by the glucose-transferring enzyme of *P. saccharophila*. This supports the view that the extracts of *C. kluyveri* contain a *trans*-acetylase.

This is not, however, the only possible explanation of the results. The arsenolysis of acetyl phosphate and the phosphate exchange could also occur if acetyl phosphate were reversibly reduced to acetaldehyde and inorganic phosphate. This explanation is not very attractive in view of the fact that no reducing agent was added in the above experiments. It could be argued, however, that such a reduction was catalyzed by trace amounts of a reduced coenzyme already present in the enzyme preparation (Reaction 14).



Since arseniate can substitute to a limited extent for inorganic phosphate in the oxidation of acetaldehyde (21), such a reversible reduction of acetaldehyde could explain the arsenolytic action on acetyl phosphate as well as the phosphate exchange.

During the course of studies with various enzyme preparations of *C. kluyveri*, one preparation, the cell-free extract of Lot E, was found to be completely incapable of catalyzing the oxidation of acetaldehyde. However, this preparation could catalyze the rapid arsenolytic decomposition of acetyl phosphate (Fig. 1). Thus the possibility that arsenolysis occurs by means of a reversible reduction of acetyl phosphate by Reaction 14 appears to be excluded.

One other possible mechanism for the arsenolysis reaction should be mentioned; namely, a reversible condensation of acetyl phosphate with acetate (Reaction 15).



That such a condensation of acetyl phosphate and acetate must occur is evident from the fact that acetyl phosphate and acetate can be reduced to butyrate. It has not been established, however, that the primary condensation results in the liberation of inorganic phosphate.

Of some interest in this connection are studies by Lipmann and Tuttle (16) with enzyme preparations of *C. butylicum* and *Escherichia coli*. Their preparations also catalyzed a rapid exchange between acetyl-

bound phosphate and inorganic phosphate. In view of the fact that enzyme preparations of *C. butylicum* catalyzed the breakdown of pyruvate to acetyl phosphate, CO_2 , and H_2 (Reaction 16), while the enzyme preparation of *E. coli* catalyzed the phosphoroclastic splitting of pyruvate to acetyl phosphate and formate (Reaction 17), Lipmann and Tuttle concluded that the phosphate exchange was due primarily to a reversibility of these reactions.



Noteworthy, however, was the fact that the rates of phosphate exchange in both systems were not influenced significantly by the addition of reactants other than acetyl phosphate, *e.g.*, hydrogen, CO_2 , or formate. In view of this situation, it would seem that their conclusion that the phosphate exchange was due mainly to a reversibility of Reactions 16 and 17 is inadequately supported. An alternative explanation would be that the exchange was catalyzed by a *trans*-acetylase according to Scheme II.

DISCUSSION

The evidence presented in the experimental section demonstrates clearly that the theory of caproic acid formation described in the introduction is largely incorrect in spite of the fact that three of the four postulated reactions are catalyzed by enzymes derived from *C. kluyveri*. Reaction 1, the oxidation of ethanol to acetyl phosphate, certainly participates in caproic acid synthesis. Reaction 2, the transphosphorylation between acetyl phosphate and the adenylic acid system, can occur and it may participate in the utilization of phosphate bond energy for the synthesis of cellular constituents, but it is not essential for butyric or caproic acid synthesis. Reaction 3, the transphosphorylation between adenosine triphosphate and butyrate, is also catalyzed by the enzyme system, but it does not appear to have any physiological significance. Indeed, the evidence we have obtained supports the view that butyryl phosphate does not participate in the synthesis or oxidation of caproic acid (Reaction 4) and is not formed in significant quantities during the normal metabolism of *C. kluyveri*. The latter conclusion is based on the observation that butyl alcohol, the main product of butyryl phosphate reduction by the enzyme preparation, is not formed by living bacteria from ethyl alcohol and acetate, whereas propyl alcohol is formed in quantity from ethyl alcohol and propionate.

The virtual exclusion of butyryl phosphate as a precursor of caproic acid opens the possibility that the reactants in the $\text{C}_4\text{-C}_2$ condensation

are butyrate and acetyl phosphate. If this is correct, the presence of the phosphoryl group in acetyl phosphate must activate the methyl as well as the carboxyl group.

The enzyme that causes the arsenolytic decomposition of acetyl phosphate is of special interest because it appears to catalyze a transfer of acetyl groups from one acceptor to another. The only acceptors so far identified are phosphate and arsenate.³ However, in view of the obvious importance of C_2 unit condensations in fatty acid synthesis, it seems likely that the transacetylating enzyme also reacts with organic acceptors to form products directly involved in elongation of the carbon chain.

It has long been suspected, on the basis of indirect evidence, that butyl alcohol is formed in bacteria by reduction of butyric acid or a derivative. The conversion of butyric acid to butyl alcohol was clearly demonstrated by Wood *et al.* (28) using C^{13} as a tracer. The direct reduction of the free carboxyl group was unlikely, because such a reaction requires a very powerful reducing system. Following the discovery of the carboxyl phosphate compounds (19, 13) Lipmann recognized that the reduction of butyric acid would be greatly facilitated in a thermodynamic sense by phosphorylation of the carboxyl group. Our experiments provide the first direct evidence for the enzymatic reduction of acyl phosphates to the corresponding alcohols. However, it may be noted that this reduction is merely the reverse of the oxidation of alcohols to acyl phosphates, a reaction previously studied with enzyme preparations of *C. kluyveri* (21).

SUMMARY

Enzyme preparations of *Clostridium kluyveri* catalyze a rapid reversible transfer of the phosphoryl group of monoacetyl phosphate to adenylic acid and adenosine diphosphate. The transfer to adenylic acid appears to be direct, since no myokinase activity could be detected.

A quantitative chromatographic procedure has been developed by which acyl phosphate derivatives of the lower fatty acids (2 to 6 carbon atoms) can be estimated in a mixture. By means of this method it was shown that enzyme preparations of *C. kluyveri* catalyze a transfer of the phosphoryl group of acetyl phosphate to fatty acids of 3 to 6 carbon atoms. These transphosphorylations are not mediated by the adenylic acid system.

With molecular hydrogen as the reducing agent, acyl phosphate derivatives of propionate, butyrate, valerate, and caproate are reduced enzymatically to the corresponding alcohols. Evidence is presented that

³ Recently amino acids have been found to act as acetyl acceptors under special conditions.

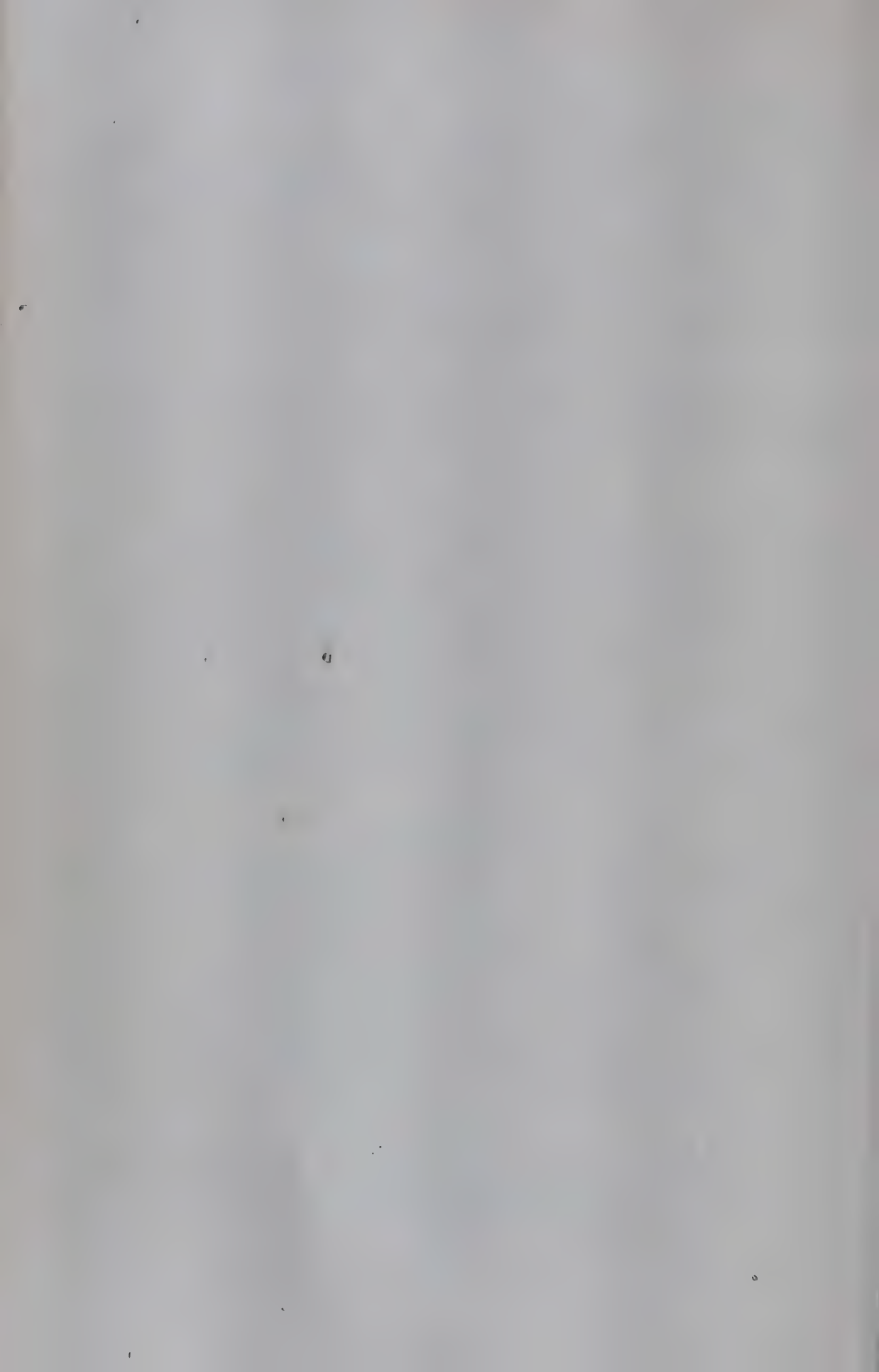
appears to eliminate butyryl phosphate from consideration as an intermediate in the synthesis and oxidation of caproate.

In the presence of arsenate, the enzyme preparations catalyze a rapid and complete hydrolysis of acetyl phosphate to acetate and inorganic phosphate. This arsenolysis reaction is specific for acetyl phosphate; arsenate has little or no influence on the rate of decomposition of other acyl phosphates. Several types of evidence indicate that the arsenolysis reaction is catalyzed by an acetyl-transferring enzyme.

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THE RELATIONSHIP OF FOLIC ACID TO FORMATE METABOLISM IN THE RAT*

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There are many indications that vitamins containing *p*-aminobenzoic acid (PABA) are concerned with the synthesis of a variety of metabolites (1-10).

The mode of action of the PABA-containing vitamins is suggested by the work of Shive *et al.* (11), who found that the amine which accumulates in sulfanilamide-treated *Escherichia coli* (12) is 5-amino-4-imidazolecarboxamide. The addition of only a single carbon atom is necessary to convert this amine to a purine. It appears that sulfanilamide has blocked the enzymatic addition of this one carbon fragment.

It now appears that a common metabolic step in the synthesis of the various metabolites which relieve sulfonamide inhibition or which stimulate growth in PABA-requiring mutant strains of *E. coli* (13) is a reaction involving formate or one of its derivatives. Several examples may be cited. The carbon of formate is known to be fixed into carbon atoms 2 and 8 of the purine-uric acid (14) (compare the interrelation between folic acid and purines (1, 2, 4, 5)). Serine, which is effective in the partial reversal of sulfanilamide inhibition (6), has been shown by Sakami (15) to be formed in the rat from formate and glycine. Furthermore Holland and Meinke (16) and Broquist and Snell (17) have shown that folic acid promotes the synthesis of this amino acid in bacteria. Glycine in which the α -carbon gives rise to formate (18) stimulates the growth of PABA-less mutants of *E. coli* (13).

Several other examples might be cited in which the evidence for this same interrelationship is suggested but is less well established. As a means of learning more about the function of PABA-containing vitamins, we have investigated the effect of folic acid on the metabolism of formate in the rat.

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In this paper data are presented on this relationship as it affects certain amino acids and heme.

EXPERIMENTAL

Weanling Sprague-Dawley rats were raised on a purified casein-sucrose diet without added folic acid and containing 2 per cent succinylsulfathiazole (Table I). The level of crystalline vitamins in this diet was 4 to 5 times higher than that usually employed to assure that these factors would not be limiting. After 5 weeks on this ration, six rats which showed typical symptoms of folic acid deficiency such as lack of gain in weight and depressed leucocyte count were selected for experimentation. Three of the deficient rats were given folic acid¹ for 4 days and pair-fed with three deficient animals during the period of treatment. A remission of the leu-

TABLE I
Ration Components (Weight per Kilo)

220 gm.	Casein, vitamin-free*	6 mg.	Pyridoxine·HCl
685 "	Sucrose	60 "	Ca pantothenate
45 "	Salts IV†	100 "	<i>p</i> -Aminobenzoic acid
50 "	Corn oil (Mazola)	20 "	Menadione
20 "	Succinylsulfathiazole	0.3 mg.	Biotin
6 mg.	Thiamine·HCl	2 gm.	Inositol
12 "	Riboflavin	4 "	Choline
40 "	Niacin		

* General Biochemicals, Inc.

† Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).

copenia was observed in the folic acid-treated animals, indicating that a physiological response (19) to the vitamin had occurred (Table II). Hereafter the folic acid-treated animals will be considered the "controls."

HC¹⁴OOH prepared from BaC¹⁴O₃, according to Melville *et al.* (20), was injected intraperitoneally into deficient and folic acid-treated animals. The three animals in each group were placed in a chamber and respiratory carbon dioxide was collected for three 1 hour periods.

Counts of radioactivity were made with a Q gas counter with dry samples (finely pulverized and dried with heat from an alcohol slurry) mounted on flat bottomed aluminum dishes. Reported values (net counts) have been corrected for background and self-absorption (21) by using the self-absorption curve obtained for BaCO₃. The error introduced by using this correction for organic compounds is relatively small (22) and, since the im-

¹ Doses listed in Table II.

portant comparisons here were made between equal weights of the same compound, it does not influence the interpretations of the data. Sufficient counts were made to establish an accuracy of about ± 5 per cent (22) for all samples except certain degradation fractions which exhibited weak specific activity.

TABLE II
Effect of Folic Acid on Leucocyte Count

Rat No.	Counts per c.mm. before treatment	Treatment	Counts per c.mm. after 4 day test period
31	3,800	None	5,500
41	7,800	"	8,000
4	3,450	"	2,300
2	8,500	+ folic acid*	28,000
5	7,700	+ " "	26,000
28	9,200	+ " "	21,500
Control (diet with folic acid)	33,000	None	33,000

* Folic acid was administered at a level of 1 mg. orally and 0.1 mg. intraperitoneally per rat per day.

TABLE III
C¹⁴ Content of Respiratory CO₂ of Rats Given Single Intraperitoneal Injection of C¹⁴-Formate

After injection <i>hrs.</i>	Activity as net counts per sec. per gm. BaCO ₃		C ¹⁴ of respiratory CO ₂ as per cent administered dose	
	Control	Low folic acid	Control	Low folic acid
1st.....	27,100	9,700	12.0	5.2
2nd.....	22,200	12,100	10.0	6.8
3rd.....	6,750	9,200	3.0	4.7
Total.....			25.0	16.7

Dosage of C¹⁴ administered, 2 mg. of formic acid (160,000 c.p.s.) per 100 gm. of body weight.

During the 1st hour period the respiratory carbon dioxide of the controls contained more than twice as much C¹⁴ as that of the deficient rats (Table III). During the 2nd hour this difference was less than 2-fold. The level of respiratory C¹⁴O₂ decreased considerably in both groups during the 3rd hour and the relative amounts of C¹⁴O₂ excreted were about equal.

Protein of liver and viscera (kidney, spleen, pancreas, heart, testes, and small intestines) was isolated by treatment with cold trichloroacetic acid.

to remove acid-soluble materials. The nucleic acid fraction was then extracted from the residue with hot trichloroacetic acid solution (23). Lipids were extracted from the proteins with a 3:1 ether-ethanol mixture. A count of infinitely thick layers of these preparations revealed that the liver protein of the treated control rats contained more than 10 times as much radioactivity as did that of the folic acid-deficient rats (Table IV). In the respective visceral protein samples the difference was about 3-fold.

The proteins were hydrolyzed by refluxing with 8 N sulfuric acid for 18 hours. After neutralizing with barium hydroxide, some of the amino acids were isolated by conventional methods (24). The heme and all amino acids reported here were recrystallized from three to eight times until each specimen showed constant radioactivity. In addition the amino acids were chemically pure within the limits of the assay methods described below.

TABLE IV
Incorporation of Radioactive Carbon of Formate into Tissue Proteins

Tissue	Treatment	Radioactivity counts per sec.*
Liver	Folic acid	1.18
"	None	0.09
Viscera	Folic acid	59.4
"	None	20.6

* These samples were counted as infinitely thick layers. The counts reported for liver and visceral protein are not comparable, since the former was counted with an end window counter and the latter with a Q gas counter.

Tyrosine was obtained by isoelectric precipitation at pH 6. The dicarboxylic acids were separated as the barium salts. Glutamic acid was isolated as the hydrochloride and aspartic acid as the copper salt. The copper salts were dried *in vacuo* at 56° over P₂O₅ to free them of water of crystallization. The purity of aspartic acid was ascertained manometrically by treatment with chloramine-T (25). After removal of the acid amino acids, the basic amino acids were precipitated by phosphotungstic acid. Histidine from this fraction was purified through the silver and mercury salts and finally as the nitranilate. After the removal of histidine, arginine was precipitated from the silver salt solution by raising the pH from 7.4 to 10 to 12 with barium hydroxide. The silver salt of arginine was converted to arginine monoflavinate for counting. For degradation studies this salt was converted to the hydrochloride, the purity of which was determined manometrically by treatment with arginase and urease (26). Two of the monocarboxylic acids were obtained from the neutral amino

acid fraction by a modification of the method of Stein *et al.* (27). Glycine was purified as the 5-nitronaphthalene-1-sulfonate. Its purity and identity were established with the phthaldialdehyde reagent of Patton (28). Azobenzenesulfonic acid was added subsequently and a precipitate, presumably the salt of alanine, was removed. Serine was then obtained by repeated fractional crystallizations of the *p*-hydroxyazobenzene sulfonate at 10° and -5°. The serine salt was enriched and finally obtained pure in the fraction which separated at -5°. The identity of serine was established microbiologically² (29) after regeneration of the free acid from the

TABLE V

C¹⁴ Fixed into Amino Acids of Folic Acid-Treated and Deficient Rats

Tissue	Compound	Isolated and counted as	Activity, net counts per sec. per gm.	
			Control	Low folic acid
Liver	Serine	<i>p</i> -OH-azobenzene sulfonate	3,640	0
	"	Free acid	12,300	0
	Glutamic and aspartic	Barium salts	1,450	270
	Arginine	Flavianate	290	130
	Glycine	5-Nitro-1-sulfonate	32	5
	Tyrosine	Free acid	0	0
	Histidine	Nitranilate	2	0
Viscera	Serine	<i>p</i> -OH-azobenzene sulfonate	4,500	1300
	Glutamic acid	Hydrochloride	1,460	490
	Aspartic "	Copper salt	1,000	360
	Arginine	Flavianate	115	40
	Glycine	5-Nitro-1-sulfonate	90	60
	Tyrosine	Free acid	50	0
	Histidine	Nitranilate	0	0
	Heme	Hemin	240	0
Blood				

salt. Its purity was ascertained by oxidation with periodate and determination of the resulting formaldehyde (30, 31).

RESULTS AND DISCUSSION

The *C¹⁴* contents of the isolated amino acids are summarized in Table V. The most striking influence of folic acid is exhibited by the liver serine. Serine isolated from the control liver protein contained a very high concentration of *C¹⁴*, while that from the deficient rats contained no detectable radioactivity. The serine isolated from the visceral protein of the defi-

² We wish to thank Dr. Betty Steele for this determination.

cient rats did contain an appreciable amount of C^{14} , but the corresponding compound from the folic acid-treated control rats contained more than 3 times as much.

The folic acid-treated animals also fixed a greater amount of C^{14} into glutamic and aspartic acids, arginine, and glycine than did the deficient rats. The differences between the control and deficient animals in radioactivity of these amino acids were greater in the case of the specimens isolated from the liver than in those isolated from the pooled viscera proteins.

The serine samples were degraded with periodate.³ Practically all of the radioactivity was located in the β -carbon atom (Table VI), which is in agreement with the results of Sakami (15). Similar results were obtained with the liver serine from the control group; however, technical difficulties prevented an absolutely quantitative determination and the counts are therefore not reported here.

In similar experiments with biotin-deficient rats and their pair-fed controls, 0.5 mg. of formic acid (40,000 c.p.s.) was administered per 100 gm.

TABLE VI
Distribution of C^{14} in Visceral Serine

Diet of rats	Counts $\times 10^{-3}$ per sec. per mole serine			
	Serine	—COOH	α -Carbon	β -Carbon
1. Folic acid.....	1730	4	3	1740
2. Deficient.....	500	1	1.5	480

of body weight. The serine from the livers of the control rats and the biotin-deficient rats had an activity of 3900 and 3400 c.p.s. per gm. of serine respectively.

Visceral aspartic acid was degraded by treatment with chloramine-T (25), which splits off both carboxyl groups. While the radioactive carbon content of the carboxyl groups of aspartic acid from the control rats was

³ 3 ml. of 0.5 M periodic acid are added to 10 ml. of 0.1 M phosphate buffer (pH 5.8) containing 10 to 20 mg. of serine. The mixture is boiled gently and the formaldehyde (β -carbon) is distilled into a receiver, while a slow stream of nitrogen is passed through the apparatus. The carbon dioxide, which is derived from the carboxyl group by this treatment, is trapped in a tube containing barium hydroxide solution which is attached beyond the receiver. About three-fourths of the original liquid volume is distilled during the 15 minute boiling period. 15 ml. of saturated dimedon solution are added to the formaldehyde-containing distillate and the precipitate is allowed to form during a 48 to 72 hour period at room temperature (30). The still residue contains the formic acid derived from the α -carbon. Formic acid is converted to carbon dioxide by oxidation with mercuric chloride (32) and the carbon dioxide is trapped in $Ba(OH)_2$ and counted as $BaCO_3$.

wice as large as that from the deficient ones, this difference was more than 2-fold for carbons 2 and 3 (Table VII). Metabolic pathways are known by which the β -carbon of serine can pass into carbon 2 or 3 of aspartic acid (33); this may account for the more pronounced effect of folic acid on the incorporation of the carbon of formate into these positions. The C^{14} in the carboxyl groups of aspartic acid is probably derived from carbon dioxide.

Upon decarboxylation of glutamic acid with chloramine-T (25) a comparable situation is encountered (Table VIII). While the ratio of the specific activity for the 1-carboxyl of glutamic acid of the control *versus* the

TABLE VII
Distribution of C^{14} in Visceral Aspartic Acid

Diet of rats	Counts per sec. $\times 10^{-3}$ per mole copper aspartate		
	Copper aspartate*	—COOH groups	Carbons 2 + 3 (by difference)
1. Folic acid.....	190	100	90
2. Deficient.....	70	50	20
Ratio of (1):(2).....	2.7	2.0	4.5

* $C_4H_5O_4NCu$.

TABLE VIII
Distribution of C^{14} in Visceral Glutamic Acid

Diet of rats	Counts $\times 10^{-3}$ per sec. per mole glutamic acid		
	Glutamic acid	1-Carboxyl	4-Carbon residue (by difference)
1. Folic acid.....	270	45	225
2. Deficient.....	90	21	69
Ratio of (1):(2).....	3.0	2.1	3.3

deficient rats is about 2:1, this ratio is larger for the remaining 4-carbon moiety. The C^{14} in the 1-carboxyl of glutamic acid can be derived from carbon dioxide via the Krebs tricarboxylic acid cycle.

On a molar basis the C^{14} content of visceral glutamic acid was 20 to 30 per cent higher than that of the corresponding aspartic acid. This phenomenon could perhaps be explained by the formation of methyl-labeled acetate from serine via pyruvate (33). This C_2 fragment might then condense with already labeled oxalacetate. The resulting tricarboxylic acid could be transformed to glutamic acid by known pathways and would contain more C^{14} -labeled carbon atoms than the aspartic acid.

The C^{14} content of the guanidino group of arginine was determined by

treating this amino acid with arginase and urease (26). There was about half as much C^{14} in the guanidino groups of arginine from the deficient animals as in that from the controls (Table IX). It has been shown by Delluva and Wilson (34) that the carbon of carbon dioxide is inserted into the guanidino group of arginine. Therefore, it is of interest here that the difference of incorporation of C^{14} between samples from the two groups of animals is of a similar order of magnitude in the guanidino group of arginine, the carboxyls of aspartic acid, the 1-carbon of glutamic acid, and the respiratory carbon dioxide. This difference (approximately 2:1 in all cases) undoubtedly arises as a result of the more rapid conversion of formate to CO_2 in the folic acid-treated animals. It is possible that part of this conversion takes place only after formate has been fixed into serine and the latter degraded through pyruvate and "acetate" by way of the Krebs cycle.

TABLE IX
Distribution of C^{14} in Arginine

Treatment	Counts $\times 10^{-3}$ per sec. per mole arginine		
	Arginine*	Guanidino group	Ornithine moiety (by difference)
Folic acid (liver)	170	125	45
Deficient "	65	70	-5
Folic acid (viscera)	55	31	24
Deficient "	17	15	2

* Counted as arginine hydrochloride after regeneration from the flavianate. The counts per mole of arginine agreed within experimental error with those obtained on the flavianate.

The relatively greater fixation of $C^{14}O_2$ by the rat from administered C^{14} -formate (Table V) than from C^{14} -bicarbonate under the same conditions (35) can be explained by the assumption that formate is converted to CO_2 within the cells where it can be fixed. Intraperitoneally administered C^{14} -bicarbonate probably is excreted so rapidly that an appreciable concentration is not accumulated by the cells from the circulating fluids.

The influence of folic acid on the radioactive carbon content of the ornithine moiety of arginine is much more pronounced than on that of the guanidino group. This phenomenon is reminiscent of the previously described situation in the case of carbons 2 and 3 of aspartic acid and the 4-carbon residue of decarboxylated glutamic acid, and a similar explanation can be offered, particularly since a metabolic interrelationship of ornithine and glutamic acid in the rat has been established (36).

The visceral tyrosine of the control animals exhibited a small but consistent radioactivity, even upon recrystallizing six times. To eliminate the

possibility that the activity of the tyrosine might result from coprecipitated cystine, the tyrosine was recrystallized in the presence of sulfite, which would convert cystine to soluble cysteine; however, the specific activity of the tyrosine remained unchanged.

The histidine of both liver and viscera was not significantly radioactive.

Hemin was isolated (37) from blood pooled from the three rats in each lot. There was an appreciable incorporation of the carbon of formate into the heme of the control animals but none into that of the deficient animals (Table V). The deficient rats were not anemic. One cannot tell from this experiment whether this is a direct insertion of formate into the porphyrin molecule or whether heme is synthesized from a molecule into which formate carbon was previously incorporated. There are several possible pathways by which formate might be incorporated into heme.

Acetate labeled in the methyl group could be formed from β -tagged serine by way of pyruvate (33). The acetate could then be utilized in the formation of heme, as shown by Bloch and Rittenberg (38).

The amino group and the α -carbon of glycine and serine are known to be inserted into heme (39-41), while the carboxyl is not. It is not known, however, whether the ethanolamine *portion* of serine is utilized for heme formation. Muir and Neuberger (42) have shown recently that ethanolamine itself is not thus used to any appreciable extent.

Formate may be directly incorporated into heme. For example, it would require the insertion of only 1 carbon into certain bile pigments to form a porphyrin.

It is not clear *where* such an incorporation of the radioactive metabolite could take place. Not enough information is available to know whether, during the 4 day period of folic acid treatment of our deficient rats, an adequate number of new, relatively immature red blood cells could be formed which are still synthesizing hemoglobin when admitted into the blood stream. The incorporation of C^{14} into the heme of the control rats is so large that it seems questionable whether one can account for it on the basis of synthesis of heme in the bone marrow during the 3 hour experimental period.

At any rate it is apparent that folic acid is implicated in a mechanism or mechanisms which are involved in the insertion of the carbon of formate into heme and it is interesting in this connection that a hematopoietic response has been obtained with folic acid in patients with anemia (43) and in laboratory animals with anemia of dietary origin (44).

SUMMARY

Rats were made deficient in folic acid by feeding a purified diet containing 2 per cent succinylsulfathiazole. Three deficient rats were given folic

acid for 4 days while being pair-fed with three other deficient animals. All were then given HC^{14}OOH intraperitoneally at a level of 160,000 c.p.s. per 100 gm. of body weight and were sacrificed 3 hours later.

Folic acid-treated rats fixed about 10 times as much C^{14} into liver protein and 3 times as much into viscera protein as did the deficient rats.

Amino acids were isolated from these proteins and it was found, in agreement with Sakami, that the carbon of formate is predominantly incorporated into the β -carbon of serine. The specific radioactivity of the β -carbon of serine of the folic acid-treated animals was much larger than that of those not treated. Similar differences in C^{14} incorporation were also found in fractions containing aspartic and glutamic acids, arginine and glycine.

The difference in C^{14} content between samples of the two groups of animals is approximately 2:1 for the 1-carbon atom of glutamic acid, the carboxyls of aspartic acid, the guanidino group of arginine, and the respiratory CO_2 , indicating a more rapid conversion of formate to CO_2 in the folic acid-treated animals. The influence of folic acid treatment on the radioactivity was still more pronounced on carbon atoms 2 and 3 of aspartic acid, the 4-carbon residue of decarboxylated glutamic acid, and the ornithine portion of arginine. The effect of folic acid on the fixation of C^{14} into these moieties is probably the result of its effect on the incorporation of C^{14} -formate into the β -carbon atom of serine.

Heme from the blood of the folic acid-treated rats contained an appreciable quantity of C^{14} ; that from the deficient rats contained none.

The incorporation of C^{14} from formate into the β -carbon of serine was not influenced by biotin deficiency in rats.

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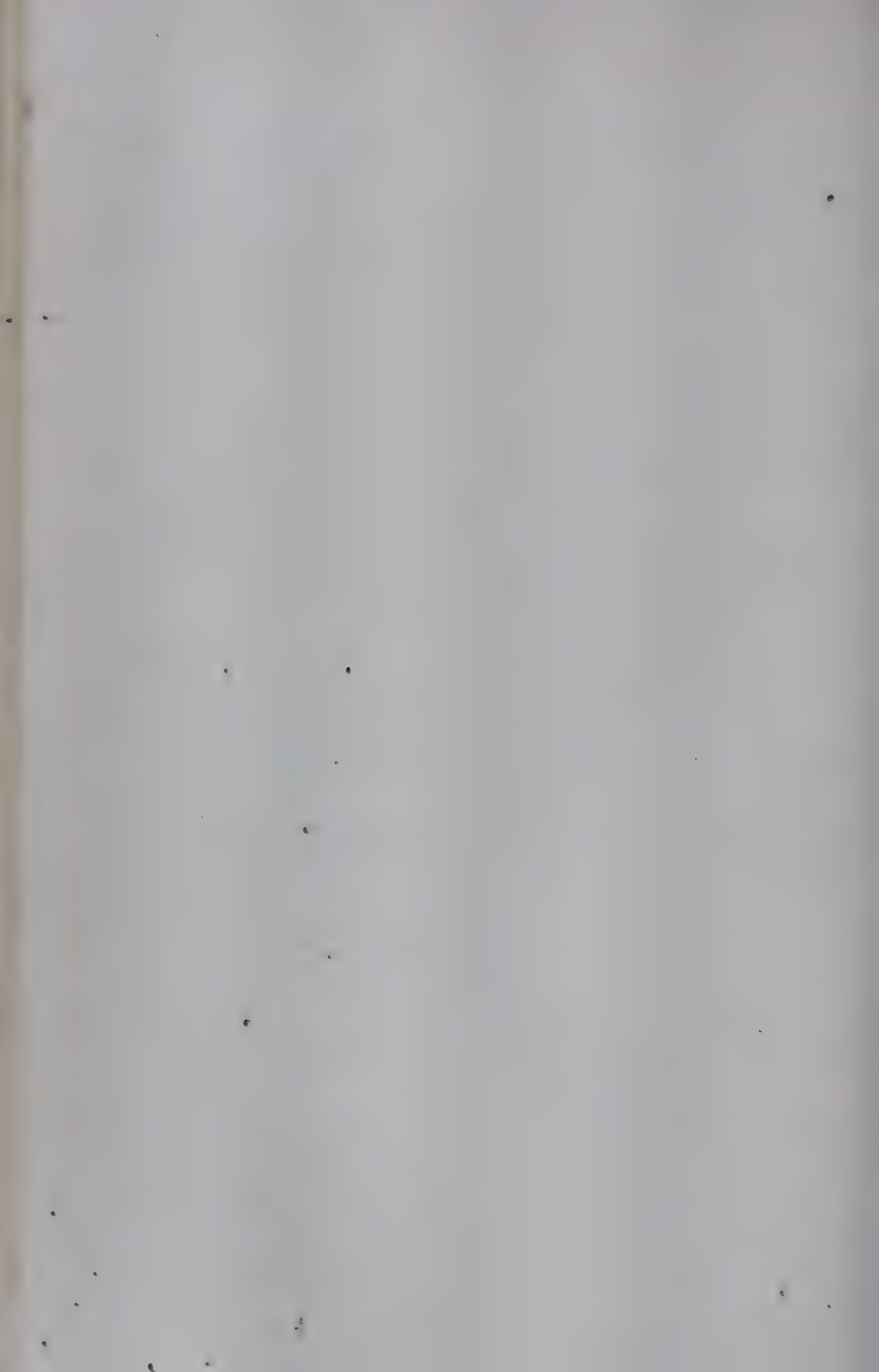
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